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AD-A217 307

The Molecular Targets of Selected Organophosphorus Compounds at Nicotinic, Muscarinic, GABA, and Glutamate Synapses: Acute and Chronic Studies Including Prophylactic and Therapeutic Approaches

ANNUAL AND FINAL REPORT

November 28, 1988

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Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4219

University of Maryland School of Medicine
Baltimore, Maryland 21201

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90 01 29 012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	**1b. RESTRICTIVE MARKINGS**
2a. SECURITY CLASSIFICATION AUTHORITY	**3. DISTRIBUTION/AVAILABILITY OF REPORT** Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	**5. MONITORING ORGANIZATION REPORT NUMBER(S)**

6a. NAME OF PERFORMING ORGANIZATION University of Maryland School of Medicine	**6b. OFFICE SYMBOL** *(If applicable)*	**7a. NAME OF MONITORING ORGANIZATION**
6c. ADDRESS (City, State, and ZIP Code) Baltimore, MD 21201		**7b. ADDRESS (City, State, and ZIP Code)**
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command	**8b. OFFICE SYMBOL** *(If applicable)*	**9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER** DAMD17-84-C-4219

8c. ADDRESS (City, State, and ZIP Code)

Fort Detrick
Frederick, Maryland 21701-5012

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO. 3M1-	TASK NO.	WORK UNIT ACCESSION NO.
61102A	61102BS11	AA	056

11. TITLE (Include Security Classification)
The Molecular Targets of Selected Organophosphorus Compounds at Nicotinic, Muscarinic, GABA and Glutamate Synapses: Acute and Chronic Studies Including Prophylactic and Therapeutic Approaches

12. PERSONAL AUTHOR(S)
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13a. TYPE OF REPORT Annual and Final Rpt.	**13b. TIME COVERED** FROM 9/15/84 TO 9/14/88	**14. DATE OF REPORT (Year, Month, Day)** November 28, 1988	**15. PAGE COUNT** 58

16. SUPPLEMENTARY NOTATION
Annual covers period of time Sept. 15, 1986-August 31, 1988.

17. COSATI CODES

FIELD	GROUP	SUB-GROUP
06	15	
06	04	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
cholinesterase; organophosphorus agents; acetylcholine; nicotinic receptor-ion channel complex; desensitization; oximes; irreversible cholinesterase inhibitors; (u=)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The major goals of this project have been: a) to continue our investigation of organophosphorus compounds (OPs) on chemosensitive receptors located at various synapses; b) to attempt to discern the active sites of the receptor-ion channel complexes involved in activation, desensitization and blockade; c) to establish the concentration gradients for various noncompetitive antagonists for production of states of activation, inactivation and blockade of the receptor-channel macromolecule; d) to delineate avenues of testing, in *in vivo* and *in vitro* conditions, new agents which may be proven effective in alleviation of symptomatology and intoxication produced by OPs; e) to recommend an agent or combination of agents which will be prophylactically and therapeutically effective against OP intoxication.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	**21. ABSTRACT SECURITY CLASSIFICATION** Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller	**22b. TELEPHONE (Include Area Code)** 301/663-7325 **22c. OFFICE SYMBOL** SGRD-RMI-S

DD Form 1473, JUN 86   *Previous editions are obsolete.*

SUMMARY:

The major goals of this project have been: a) to continue our investigation of organophosphorus compounds (OPs) on chemosensitive receptors located at various synapses; b) to attempt to discern the active sites of the receptor-ion channel complexes involved in activation, desensitization and blockade; c) to establish the concentration gradients for various noncompetitive antagonists for production of states of activation, inactivation and blockade of the receptor-channel macromolecule; d) to delineate avenues of testing, in _in vivo_ and _in vitro_ conditions, new agents which may be proven effective in alleviation of symptomatology and intoxication produced by OPs; e) to recommend an agent or combination of agents which will be prophylactically and therapeutically effective against OP intoxication.

We have shown that noncompetitive blockade and desensitization of the peripheral acetylcholine receptor-ion channel macromolecule (AChR) are common denominators of many threat agents that may be significant both to lethality of nerve agents and to protective effects of a spectrum of antidotes. The AChR effects of carbamates, oximes, and SAD-128 modulate neuromuscular transmission in a manner which promotes therapeutic efficacy. It is likely that peripheral AChR effects are secondary to toxic effects in the CNS, where homologous ion channels of excitatory transmitter-gated receptors may be similarly affected. In fact, our studies are consistent with the notion that the AChR and NMDA-sensitive receptors on central neurons share a great degree of functional homology with AChRs in acutely dissociated muscle fibers. Thus our studies on the sensitivity of AChRs to stereoselective carbamates, mecamylamine, scopolamine and atropine using binding, patch and whole cell voltage clamp techniques are most important for delineating the mechanism and site of action of OPs in the peripheral and central nervous sytems.

FOREWORD:

Staff, with percent of effort on each project:

Alkondon, Dr. M.   8/85 to present
    Voltage and patch clamp studies, twitch, and cholinesterase studies with OPs and oximes (100%)

Aracava, Dr. Y.   10/84 to present
    Patch clamp studies involving desensitization (50%) and partial agonists (50%)

Cintra, Dr. W.M.   8/85 to present
    Electron microscopic, voltage and patch clamp studies of physostigmine (50%) and mecamylamine (50%) stereoisomers

Costa, Dr. A.C.S.   8/88 to present

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By _____
Distribution/
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D-1

Patch clamp studies on nicotinic receptors (50%) and GABA receptors in CNS (50%)

Deshpande, Dr. S.S.   4/85 to 1/89
Twitch, iontophoresis and patch clamp studies, all projects (100%)

Dube, Dr. S.   11/87 to 2/88
Twitch and contracture studies with OPs and therapeutic agents (100%)

Helrich, Dr. M.
Twitch studies with OPs and oximes (100%)

Kawabuchi, Dr. M.   10/84 to 9/86
Electron microscopic studies, OPs, physostigmine enantiomers (100%)

Landman, Dr. M.-T.   11/87 to 11/89
Patch clamp studies on cultured cells of the CNS (100%)

Marrow, Mrs. B.J.   10/84 to present
Technician, all projects (100%)

Pou, Mr. L.A.C.   12/86 to 5/87
Patch clamp studies; electronic engineer: equipment design and computer applications for all projects (100%)

Radhakrishnan, Dr. V.   7/88 to present
Patch clamp studies on glutamate receptors in the CNS (100%)

Ramoa, Dr. A.S.   7/87 to 7/88
Patch clamp studies on glutamate receptors in the CNS (100%)

Rozental, Dr. R.   10/86 to 8/89
Nicotinic agonists and antagonists, stereoisomerism (100%)

Shaw, Dr. K.-P.   10/84 to 5/88 and 8/89 to present
Voltage and patch clamp studies of OPs and physostigmine (100%)

Silveira, Dr. F.   1/88 to present
Voltage clamp studies with eseroline compounds (100%)

Swanson, Dr. K.L.   10/84 to present
Partial agonist studies (100%)

Taylor, Mrs. Inez   7/88 to present
Dishwasher, all projects (25%)

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A. INTRODUCTION:

The current therapeutic regimen for poisoning by the organophosphorus compounds (OPs) soman, sarin, VX and tabun is based on correcting the excessive muscarinic receptor activity and reactivating the phosphorylated acetylcholinesterase (AChE) enzyme. We have demonstrated that the nicotinic acetylcholine receptor/ion channel (AChR) of the periphery and most likely of the CNS is another important direct target for these threat agents. Experimental treatments including antiAChEs such as physostigmine, neostigmine and edrophonium, and oximes such as HI-6 and 2-PAM and the non-oxime antidote SAD-128, some but not all of which have antiAChE effects, also affect the nicotinic AChR through actions at agonist and allosteric sites. In fact, many lines of evidence converge from electron micrographic and electrophysiological studies to indicate that the AChE inhibition by threat agents or carbamates cannot explain all of their effects.

B. METHODOLOGY:

1. Protection studies.

Female Wistar rats (200-220 g, 3 months old) were pretreated with a mixture of pretreatment drugs intramuscularly 30 min prior to subcutaneous injection of OP. Lethality was recorded for a 24-hr period post-challenge and the surviving animals were further observed for up to 10 days.

2. Electrophysiological techniques.

Tissue preparations, solutions and drugs. Amphibians: Data were collected at room temperature (20-22°C) from sartorius or cutaneous pectoris or at 10°C from interosseal muscles of the frog, *Rana pipiens.* The physiological solution used for the frog muscles had the following millimolar composition: NaCl, 116; KCl, 2.0; CaCl$_2$, 1.8; Na$_2$HPO$_4$, 1.3; and NaH$_2$PO$_4$, 0.7. The frog Ringer's solution was bubbled with 100% O$_2$ and had a pH of 6.9-7.1. Mammalians: Preparations used were innervated and chronically denervated (7-14 days) soleus muscles or phrenic nerve-diaphragm preparations of the Wistar rat (180-200 g). The physiological solution for mammalian muscle had the following millimolar composition: NaCl, 135; KCl, 5.0; MgCl$_2$, 1.0; CaCl$_2$, 2.0; NaHCO$_3$, 15.0; and Na$_2$HPO$_4$,

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1.0. During experiments this solution was continuously aerated with 95% O_2/5% CO_2, and the pH was 7.1-7.2.

Twitch studies. Twitch studies were performed on frog sartorius muscle with sciatic nerve attached. Direct muscle stimulation was applied by platinum bipolar electrode, using supramaximal square-wave pulses of 2-3 msec duration. Indirect stimulation was applied via the sciatic nerve by a separate platinum bipolar electrode, using supramaximal square-wave pulses of 0.05-0.1 msec duration. Indirect stimulation volleys were applied at a frequency of 0.2 Hz. To study direct evoked muscle twitch, neuromuscular transmission was blocked by alpha-bungarotoxin (α-BGT) (5 μg/ml). In all experiments the sartorius muscle was allowed to equilibrate under stimulation for 20-30 min before addition of drug. For each drug concentration, the duration of exposure was chosen to allow maximal effects to be observed; the exposures ranged from 15 to 45 min, with maximal effects usually occurring 10-20 min after each drug addition.

In studies where phrenic nerve-diaphragm preparations were used, the muscles were removed from rats anesthetized with ether and were mounted in a tissue bath. After a 15 min equilibration throughout the concentration of experiments, the muscles were stimulated through the phrenic nerve, using supramaximal pulses of 0.1-msec duration at 0.1 Hz. Tetani of 4 sec duration were delivered at 20 and 50 Hz. Responses to twitch and tetanic stimulation were obtained in control, in the presence of OPs and oximes, and after wash.

Endplate current (EPC) analysis. Frog sartorius muscles with nerve attached were treated with 400-600 mM glycerol to disrupt excitation-contraction coupling. The voltage-clamp circuit was similar to that of Takeuchi and Takeuchi (1) as modified by Kuba et al. (2). The membrane voltage sequence for EPC experiments consisted of 10-mV conditioning steps made in both depolarizing and hyperpolarizing directions throughout the range of +60 to -160 mV. Occasionally, larger steps were used to check for hysteresis (for details see ref. 3). Each conditioning step was 3 sec in duration; at the end of each conditioning step, an EPC was elicited by nerve stimulation. The EPC waveforms were displayed on an oscilloscope and digitized at 10 kHz by a PDP 11/40 minicomputer (Digital Equipment Corporation, Maynard, Mass.). The rise times and peak amplitudes were obtained directly from the digitized EPC data. The decay phase (80%-20%) was fit by a single exponential (linear regression on the logarithms of the data points) from which the EPC decay time constant (τ_{EPC}) was determined.

Junctional and extrajunctional sensitivity to ACh - double-barrel microiontophoresis technique. The details of this technique have been described previously (4,5). Junctional ACh sensitivity was examined in cutaneous pectoris muscles of the frog and soleus muscles of the rat. Extrajunctional ACh sensitivity was studied in 10-day denervated soleus muscles of the rat. Both barrels of a double-barrel microiontophoretic pipette were filled with 2 M ACh. One barrel of the micropipette was used for microiontophoresis of a long (30 sec) conditioning charge to release ACh, while the other barrel was used to deliver repetitive (0.2-8 Hz) brief (50-100 μsec) charges. The position of the double-barrel micropipette was adjusted so that the 50-μsec charges applied to the ACh pipette induced a response of <1.0 msec rise time. A single intracellular microelectrode measured the transient

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membrane depolarizations due to ACh. The decrease of ACh potential amplitudes delivered at 1 Hz during the conditioning pulse and the recovery time of the amplitudes after the end of the conditioning pulse are measures of desensitization.

Isolation of muscle fibers for patch clamping. Interosseal muscles were dissected from the longest toe of the hind foot of the frog Rana pipiens in standard Ringer's solution. The procedure for isolation of single fibers was reported previously (6). Briefly, the muscles were incubated in 1 mg/ml of collagenase (type I, Sigma Chemical Co., St. Louis, Mo.) (2 hr, 21°C) and then in 0.2 mg/ml of protease (type VII, Sigma) (12-20 min) with mild agitation. Fibers were stored in bovine serum albumin (0.3-0.5 mg/ml) and used within 24 hr. Tetrodotoxin (300 nM) (Sigma) was added to all solutions used in patch clamp studies to prevent contraction of the muscle fibers.

Patch clamp recording technique. Microelectrodes of borosilicate capillary glass were pulled in two stages and heat-polished to yield micropipettes with resistance of 8-12 MΩ. For recording from frog interosseal muscle fibers, ACh and/or test drugs were diluted in HEPES-buffered solution and filtered through a Millipore filter before filling the micropipette. The recording bath was filled with HEPES-buffered solution and maintained at 10°C. Recordings were made at 10°C because the time course of the events is slower than at room temperature and this facilitates a detailed kinetic analysis. GigaΩ seals between the nonjunctional surface of the fiber membrane and the microelectrode were formed using standard techniques (7).

For CNS recordings in the outside-out configuration the external solution had the following composition (mM): NaCl, 165; KCl, 5.0; $CaCl_2$, 2; Hepes, 5; pH 7.3; 310 mOsm plus 0.3 μM TTX. The internal solution was composed of (mM): CsCl, 80; CsF, 80; CsEGTA, 10; Hepes, 10; pH 7.3; 320 mOsm. The microelectrode resistance was 2-7 MΩ. Recordings were made from neurons maintained at room temperature (19-22°C). For earlier experiments using cell-attached recording, the neurons were maintained in a physiological buffer containing (mM): NaCl, 116; KCl, 5.4; $CaCl_2$, 3.0; $MgCl_2$, 1.3; $NaHCO_3$, 26.0; NaH_2PO_4, 1.0; dextrose, 11.0; pH 7.4; 315 mOsm.

Patch clamp currents from "cell-attached" or "outside-out" patches were monitored using an LM-EPC-7 instrument (List Electronic, Darmstadt, West Germany). The signal was filtered with a fourth-order low pass Bessel filter at 3 kHz and recorded on FM magnetic tape for later analysis.

For computer analysis, the signal was digitized at 0.08 msec intervals. Analysis was done by either 11/24 or 11/40 minicomputer (Digital) or by IBM (or equivalent) microcomputer, using a maximum zero-crossing algorithm to establish baselines and channel amplitudes (8). A channel opening was counted when the current was greater than 50% of the estimated average channel current. After an opening, the channel was considered to be closed when the current decreased to less than 50% of the mean channel current.

3. Tissue preparation for quantitative light and electron microscopy. After experimental treatments, the rats were anesthetized with ether and soleus muscles from the

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left limb were removed. The slow twitch soleus muscle was selected for study because it has been reported to be more affected by sarin than the fast twitch extensor digitorum longus muscle (9). Soleus muscles were pinned loosely on a Sylgard plate and fixed immediately by immersion in 3% paraformaldehyde and 1% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.2) for 1 hr. The muscles were then washed and stored in a 0.15 M cacodylate buffer. Junctional regions were identified by reacting the whole muscle for cholinesterase (ChE) activity, using ACh iodide as the substrate (10). Identified endplate regions were cut into small pieces and the precipitate was removed by washing 2-3 days in 0.15 M cacodylate buffer. Blocks of tissues containing the motor endplate were postfixed in 1% osmium tetroxide, stained en bloc with 0.5% uranyl acetate, dehydrated and embedded in Epon 812. Semithin plastic sections (1-1.5 μm) were cut from longitudinally oriented blocks and stained with toluidine blue to locate motor endplates. Ultrathin sections prepared from these blocks were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109 electron microscope.

4. **Quantitative morphometry. Light-microscopic analysis of semithin plastic sections.** For all drug treatment groups, the area, perimeter, width and length of myopathic lesions were determined using a videocamera-equipped microscope in which the image was projected onto a digitization pad (Bioquant System, Nashville, Tenn.). A planimeter program generated a running total of trapezoid areas swept by the cursor. All measured data from each group were averaged, and the standard error of the mean was calculated. The two-tailed Student's t-test was used for statistical comparison of the data. The difference between two mean values was considered significant if the probability value (P) was found to be < 0.05.

5. **Tissue culture methods.**

The method of culturing hippocampal neurons was as described by Aracava et al. (11). Briefly, female rats (Sprague-Dawley, 16-18 days of gestation) were killed by cervical dislocation and the fetuses placed in cold physiological solution. The cerebral hemispheres were isolated and the hippocampi dissected, minced and incubated with trypsin (0.25%) for 30 min at 35.5°C. After the incubation period, the medium was changed to modified Eagle's medium (MEM, Gibco, Grand Island, NY) with 10% horse serum, 10% fetal calf serum, glutamine (2 mM) and DNase (40 μg/ml). The neurons were dissociated by trituration with a Pasteur pipette and plated in a final concentration of 350,000 cells/ml in Petri dishes previously covered with astrocytes isolated from DUB mice (12). 24 hr later the culture medium was changed to MEM plus 10% horse serum and glutamine (2 mM). 1 week after plating, FDUR (53 μM) was added to avoid background cell proliferation, and after 24 hr the medium was changed to MEM plus 10% horse serum and glutamine (2 mM). The medium was changed twice a week. The hippocampal cultures were composed principally of pyramidal cells, as the granular cells were not present at this stage of development.

One- to four-week-old hippocampal cultures as young as 3 to 4 days were used for single channel recordings. The membrane potentials of these neurons were between -50 and -65 mV. In the absence of tetrodotoxin, spontaneous synaptic potentials could be recorded from all the cells tested.

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C. RESULTS AND DISCUSSION:

1. Detailed studies on OP agents.

Effect of OP agents on twitch and tetanic contractions of rat diaphragm.

Application of OP agents - soman, sarin, tabun and VX produced within 4 min a significant potentiation (20-80%) of muscle twitch (Table 1). VX was the least potent of these OP agents in augmenting muscle twitch. Values for two concentrations are given, but the facilatatory effect is already near maximal at the lower concentration. Facilitation of muscle twitch was soon followed by depression. Contractile tension recorded at 15 min after addition of an OP agent was reduced to 36-69% of control. Prolonged washing of the diaphragm muscles (up to 3 hr) produced a partial recovery (53-79% of control) of muscle twitch response.

TABLE 1

Effect of OP Agents on Twitch Tension of the Rat Diaphragm

Treatment and Dose (μM)	Twitch Tension (% Control)		
	1-4 min[a]	15 min	3 hr wash
None	100	100	88 $\pm$ 12
Soman			
0.1	161 $\pm$ 16[b]	35 $\pm$ 4	67 $\pm$ 17
0.2	171 $\pm$ 25	55 $\pm$ 18	79 $\pm$ 9
Tabun			
0.2	151 $\pm$ 5	69 $\pm$ 7	74 $\pm$ 4
0.4	158 $\pm$ 7	60 $\pm$ 6	67 $\pm$ 3
VX			
0.1	122 $\pm$ 14	58 $\pm$ 7	66 $\pm$ 21
0.2	116 $\pm$ 15	41 $\pm$ 11	53 $\pm$ 13
Sarin			
0.2	133 $\pm$ 17	42 $\pm$ 13	55 $\pm$ 1
0.4	167 $\pm$ 17	57 $\pm$ 12	72 $\pm$ 12

[a] The largest twitch recorded within the first 4 minutes after OP application is reported, as a measure of potentiation.

[b] Values are means $\pm$ S.E. recorded from 3 muscles.

OP agents had profound effects on the peak tetanic tension and on the ability of muscles to sustain tension during tetanus (Fig. 1 and Table 2, see also Table 4). The results of experiments where the phrenic nerve was stimulated at frequencies varying from 0.1 to 50 Hz for 4 sec duration for recording tetanic contractions of diaphragm in control condition, in the presence of 0.2 μM soman, and after washing (3 h) are shown in Fig. 1. Under control conditions, the peak contractile tension of the muscle increased approximately twofold at 20 Hz and fourfold at 50 Hz. In the presence of soman (0.2 μM) the peak developed at the beginning of tetanus was significantly depressed at all frequencies, but the effect was much more pronounced at higher frequencies (20 and 50 Hz). The peak tension developed at the start of tetanus recovered at levels comparable to control up to 10 Hz and only a partial recovery was observed at 20 and 50 Hz. The values for the peak tetanic tension for all the OP agents tested are shown in Table 2. The ability of the muscles to sustain tetanus was determined by measuring the muscle tension at the start and at the end of tetanic stimulation during each 4-sec test. The ratio of final/initial tension developed at each frequency in the presence of soman is shown in Fig. 1. It is obvious that, beginning at 1 Hz, the muscles are incapable of maintaining tension during

TABLE 2

Effect of OP Agents on Tetanic Tension at 50 Hz

Treatment and Dose (μM)	Peak Tetanic Tension (g)	
	After 15 min Exposure to OP	After 3 Hr Wash
None	67 ± 2[a]	61 ± 3
Soman		
0.1	9 ± 1	22 ± 5
0.2	7 ± 1	30 ± 3
Tabun		
0.2	23 ± 6	37 ± 4
0.4	12 ± 1	34 ± 5
VX		
0.1	13 ± 3	78 ± 31
0.2	7 ± 1	49 ± 13
Sarin		
0.2	11 ± 4	79 ± 26
0.4	11 ± 2	79 ± 14

[a] Values are means ± S.E. recorded from 3 muscles.

tetanus, and that after washing there is only a partial recovery (up to 20 Hz) in this parameter. The muscles exposed to soman for 15 min and washed for 3 hr failed to sustain tetanus at 50 Hz. Like soman, all other OPs showed significant depression in these values. The ability of the muscles to sustain tetanus was completely regained by washing after removal of sarin and about 86-96% recovered by washing after removal of VX. However, with soman, VX and tabun, only partial recovery was observed.
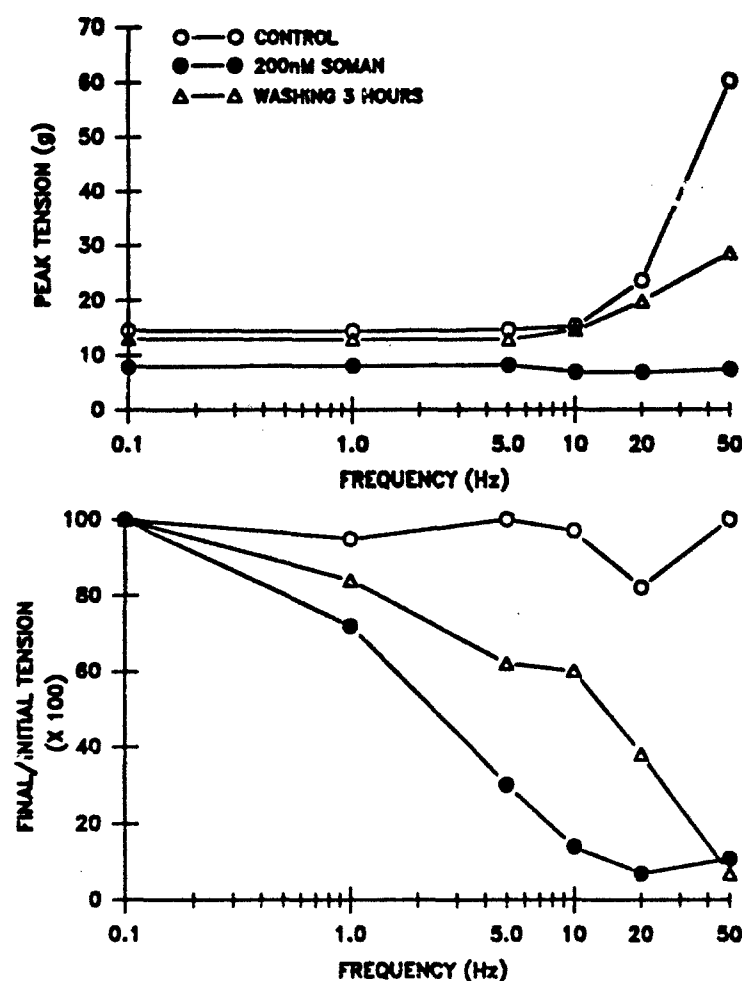
Figure 1. Effect of soman on peak tetanic tension (50 Hz) and on the ratio of final/initial tension developed in rat diaphragm muscle during 4-sec tetanus as a function of frequency. Peak tension (upper panel) or initial tension is the value shown at the beginning of tetanic stimulation. Final tension is the amount of tension developed by the muscle at the end of a 4-sec train. The ratio of final/initial tension (lower panel) is an indication of the ability of muscle to sustain tension during tetanus.

Comparative study of OP compounds on frog neuromuscular transmission.

OPs, by virtue of irreversible AChE inhibition, produce neuromuscular paralysis and subsequent respiratory arrest as toxic effects in animals and humans exposed to these agents. Earlier work from our laboratory and elsewhere indicates that both reversible and irreversible ChE inhibitors affect neuromuscular transmission by a direct action at the postsynaptic AChR as agonists, channel blockers and/or desensitizers. Though the overall effects of OPs on cholinergic synapses are known, the mechanism of interaction of these compounds at site(s) at both the pre- and postsynaptic neuromuscular junction is not well determined. The present study was undertaken to evaluate the possible multiple targets of action of the OPs soman, sarin, tabun and VX at the neuromuscular synapse, with a view to enhancing our understanding of the mechanism of their toxic effects and to finding better remedies for dealing with OP poisoning.

Experiments were conducted using frog sciatic nerve-sartorius muscle preparations for the recording of EPCs and miniature endplate currents (MEPCs). EPC peak amplitude was increased by VX, soman (both at 0.1 μM) and sarin (10 μM) but not by tabun. All four OPs decreased the peak amplitude upon further increasing the dose. The time constant of EPC decay (τ_{EPC}) was prolonged, and a maximum increase was achieved with 1 μM in the case of VX, sarin and tabun whereas soman produced a maximal increase at 0.1 μM concentration. Doses above 1 μM of all OPs shortened the τ_{EPC} from an enhanced level already achieved by a low dose. These agents also affected the MEPC in a way similar to their effects on that of the EPC. The direct interactions of VX with the postsynaptic AChR were analyzed in more detail at the single channel current level.

Single channel studies using OP agents.

Earlier work from our laboratory indicated that the organophosphate compounds soman, sarin, tabun and VX had a modulatory effect on the EPCs of frog sartorius muscles. The prolongation of τ_{EPC} by lower concentrations was antagonized by higher concentrations of these OPs. The molecular mechanism underlying the above effect was

Sarin (20 μM)
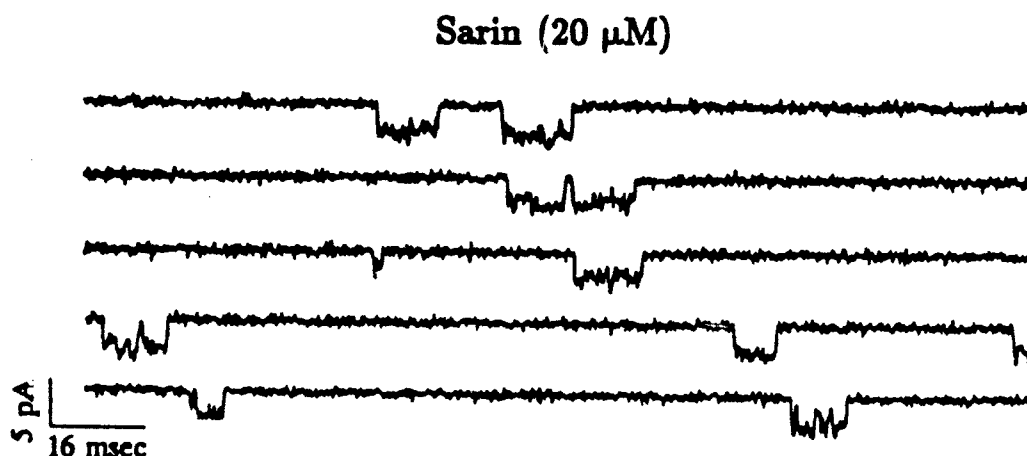
Figure 2. Samples of single channel currents activated by sarin in frog interosseal muscle. Cell attached condition, -100 mV, 10°C.

openings in the presence of OPs as compared to control condition. However, in order to address this issue more critically, studies involving higher concentrations of ACh should be done and are currently in progress.

Desensitization studies with OP agents.

Electrophysiological studies at the microscopic level with OPs soman, sarin and VX and carbamate pyridostigmine have revealed these agents to have agonist effect at the AChR with varying potencies. We were able to study desensitization more quantitatively using iontophoretic application of ACh in the denervated soleus muscles of the rat. ACh potentials were obtained by applying 100 pulses at 8 Hz. Effects of pyridostigmine, sarin and VX on desensitization in the soleus muscle are shown in Figure 3. In control conditions, the amplitude of the 100th ACh potential is decreased by at most 20% of the amplitude of the first potential. In addition, the response evoked by single pulses immediately after a train does not show any depression. In the presence of pyridostigmine or OP agents, the response of the 100th potential was depressed by as much as 52% in the presence of 20 μM sarin or by 37% in the presence of pyridostigmine, depending on the concentration of the agents. There was almost complete recovery after washing the muscles with physiological saline.

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Morphological studies.

A single sublethal dose of sarin (0.08 mg/kg) produced enlarged, blistered and severely disrupted subjunctional regions, with muscle damage extending beyond the endplate of the rat soleus muscle (Fig. 4). Myofiber necrosis, subsequent phagocytosis and regeneration of the muscle fiber over the course of the next 10 days were observed.

Molecular mechanism of OP-induced toxicity.

The peripheral component (structural and functional changes in the skeletal muscles) in the OP action has been investigated in this study. Evidence for the involvement of both AChE and AChR in the actions of OPs is found. Morphological studies indicate that the damage to the muscle structures occur as a function of the AChE-inhibitory efficacy as OPs and (-) physostigmine caused much more damage than (+) physostigmine. However there was no clear correlation between AChE activity and muscle function in presence of OPs suggesting the involvement of other sites in their action. Studies on EPCs and MEPCs indicated a direct interaction of OPs with the AChR. ACh sensitivity experiments and single channel studies confirmed a desensitizing action of OPs at the AChR. In addition, a weak agonistic activity for most of the OPs and a channel blocking effect for VX was also observed. The AChR effects (direct and indirect) appear to be involved in the peripheral toxic effect effects of OPs.

2. Carbamates and related compounds.

Morphological studies: Ability to physostigmine isomers to reduce muscle endplate lesions produced by OPs.

Quantitative light microscopic and electron microscopic (EM) data revealed that (+) physostigmine (0.3 mg/kg) induced no obvious damage in the postjunctional region, whereas (-) physostigmine (0.1 mg/kg) had a selective effect in inducing irregularities of subjunctional sarcomere band patterns without any gross vacuolization, which disappeared during the next 24 hr. Pretreatment of rats with (-) physostigmine (0.1 mg/kg) or (+) physostigmine (0.3 mg/kg) 30 min prior to injection of a lethal dose of sarin (0.13 mg/kg) had a significant effect in reducing the number and size of the lesions (Fig. 4). Lesions were detected in most endplates, but were less severe than those produced by sarin alone. Moreover, observation of muscles from pretreated rats showed complete recovery by day 5, in contrast to the 10 days required for almost complete regeneration of muscle fibers in pretreated rats injected with a sublethal dose of sarin.

In vivo studies: Protection of rats against lethality of organophosphorus (OP) agents.

We have observed that physostigmine, in comparison to pyridostigmine or neostigmine, appears to offer the highest protection against exposure to irreversible organophosphate agents (14). It has also been shown (15) that pretreatment of rats with physostigmine (0.1 mg/kg) together with atropine (0.5 mg/kg) offered almost complete

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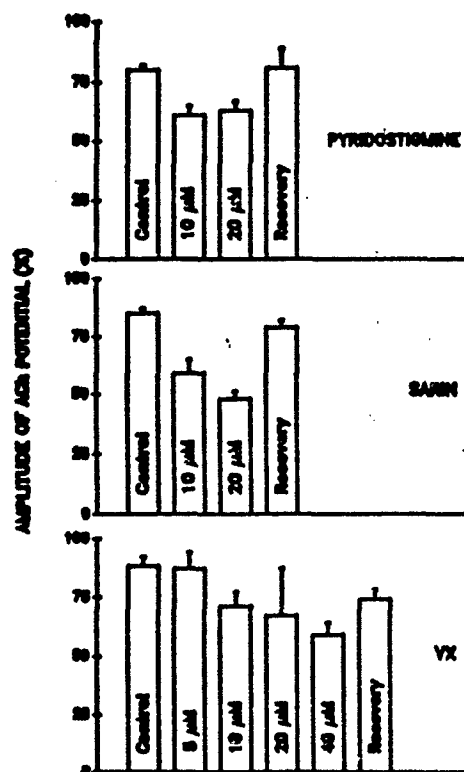
Figure 3. Effect of pyridostigmine, sarin and VX on potentials evoked by iontophoretic application of 2 M ACh in the denervated soleus muscles of the rat. The degree of desensitization is expressed as percent amplitude of the 100th potential in a train of 100 pulses evoked at 8 Hz in relation to the response to the first pulse in the same train. After control recordings, pyridostigmine or an OP was applied and 40 min was allowed for equilibration before recording in the continued presence of these agents. Recordings for recovery were obtained after 60-min perfusion with physiological saline. Values are means ± S.E. from 4 fibers.

protection to animals exposed to a lethal dose of sarin (0.13 mg/kg). Pretreatment with pyridostigmine (up to 0.8 mg/kg) or neostigmine (0.2 mg/kg) gave only 28 and 12% protection, respectively. We also showed that the combination of physostigmine and atropine (even in increased doses) does not protect rats against four to five multiples of 100% lethal dose of sarin (14). The pretreatment scheme utilizing physostigmine was also tested against lethal doses of another irreversible OP agent, VX. Subcutaneous injection of a dose of 0.015 mg/kg of VX in rats was 100% lethal. A dose of 0.05 mg/kg (about 3.5 times the lethal dose) of this agent was used. All animals receiving VX alone died within 10 min. Salivation, fasciculations, tremor, convulsions, and difficulty in breathing (gasping) were obvious symptoms of cholinergic crisis seen in these animals. Pretreatment of rats with physostigmine (0.1 mg/kg) and atropine (0.5 mg/kg) was effective in reducing VX-induced lethality to 50% (Table 3). Although the onset and severity of symptoms after VX

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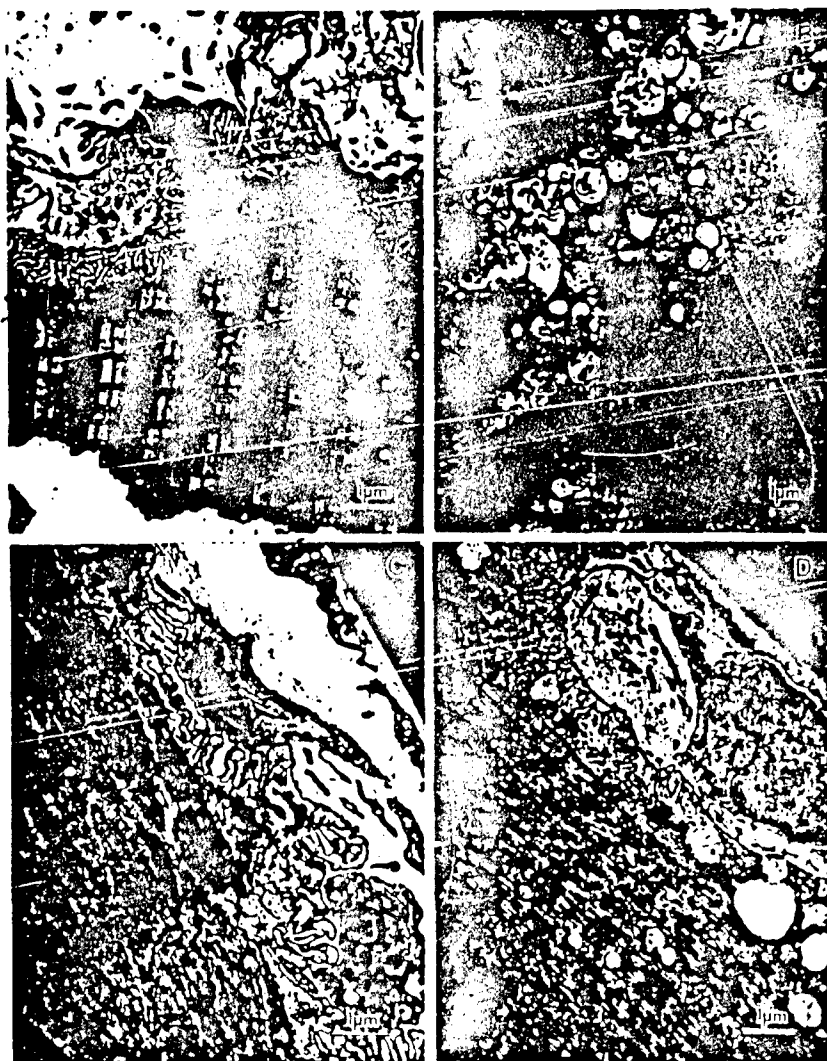
Figure 4. Protection of the motor endplate of rat soleus muscle against sarin by (+) and (-) isomers of physostigmine. A: Motor endplate and synaptic sections from a soleus muscle which was removed from a rat after it was subjected to a dose of (+) physostigmine (0.3 mg/kg). B: Motor endplate from a rat injected subcutaneously with a sublethal dose of sarin (0.08 mg/kg). The soleus muscle was removed 1 hr after injection, and longitudinally cut sections disclosed the intact motor nerve terminal. Note that the sarcoplasm is enlarged and filled with vacuoles of mitochondrial origin. The myofibrils were totally disorganized, with clear loss of the original sarcoplasmatic bands. C: Motor endplate after treatment with (-) physostigmine (0.1 mg/kg) for 1 hr. D: Motor endplate from a rat treated with (+) physostigmine (0.3 mg/kg) prior to a lethal injection of sarin (0.13 mg/kg). There was a marked decrease in myopathic lesions, with a small number of vacuoles of mitochondrial origin. Z lines showed some slight irregularities and dislocation. It is obvious that both (-) and (+) physostigmine offers significant protection against the marked damage induced by irreversible OP poisoning (16-18).

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TABLE 3

Effect of Pretreatment of Rats with Physostigmine and Ganglionic
Blockers Mecamylamine or Chlorisondamine on Protection Against Subcutaneous
Injection of Lethal Doses of VX[a]

Pretreatment[b]	Dose (mg/kg)	% Lethality[c]
None	—	100
Atropine + Mecamylamine	0.5 4.0	100
Atropine + Mecamylamine	0.5 8.0	100
Atropine + Chlorisondamine	0.5 2.0	100
Atropine + Physostigmine	0.5 0.1	50

Atropine
   + Mecamylamine
   + Physostigmine
	0.5
	4.0
	0.1
		0

Atropine
   + Chlorisondamine
   + Physostigmine
	0.5
	2.0
	0.1
		0

[a] Minimal LD100 dose of VX was 15 μg/kg. The dose used in these experiments was
50 μg/kg which represents approximately 3.5 times the LD100 dose.

[b] All the drugs used in the pretreatment were dissolved in 0.9% NaCl. The total
(intramuscular) injection volume was 0.1 ml/100 g body wt.

[c] The lethality was based on 24-hr observation in six rats per group.

administration were similar to those shown by the group of rats receiving VX alone, the
symptoms gradually subsided over the course of 4 hr. In contrast to the unprotected
group, these animals exhibited no irregularity in breathing or gasping. At 6 hr after VX,
the rats from the pretreated group showed no fasciculations or tremors; however, during
walking slight motor incoordination was evident. By 24 hr the rats appeared normal with
respect to behavior and motor ability. The dose of 0.1 mg/kg physostigmine used in this
study produced by itself hardly any symptoms other than mild fasciculations lasting for 10-
15 min. The most striking observation, however, was the fact that inclusion of one of the
ganglionic blocking drugs (mecamylamine, 4 mg/kg or chlorisondamine, 2 mg/kg) in the

pretreatment mixture of physostigmine and atropine reduced the lethality further to 0% after administration of the same dose of VX (Table 3). The onset and severity of symptoms and the pattern of recovery in these rats were similar to those shown by the animals from physostigmine- and atropine-pretreated groups. Intramuscular injection of either mecamylamine (4 mg/kg) or chlorisondamine (2 mg/kg), but without physostigmine, 30 min prior to injection of VX did not prevent the lethal actions of this agent. The only beneficial effect observed was the reduction in mucous and salivary secretions.

Interactions of physostigmine isomers with nicotinic AChR.

Agonist property. Both of the physostigmine enantiomers acted as weak agonists at muscle nicotinic AChRs. Natural (-) physostigmine was about 10-fold more potent than its optical isomer, disclosing a lower degree of stereospecificity for the agonist recognition site as compared to 40-fold potency ratio for the AChE-inhibitory site (19). The anatoxin-a enantiomeric pair, for comparison, exhibited a much higher stereospecificity for the AChr-recognition site than did the physostigmine isomers (20). However, the kinetics of the ion channels activated by physostigmine enantiomers were markedly distinct. (-) Physostigmine (>0.5 μM) activated currents that showed a high frequency of flickers during the open state of the channel (21) (Fig. 5). The presence of many fast flickers, which were too brief to be adequately recorded considering our filter bandwidth and digitization rate, contributed to a broader noise level during the channel open state and most likely accounts for the apparent decrease of single-channel conductance observed in the presence of (-) physostigmine recordings. In contrast to (-) physostigmine recordings, brief, square, well-separated pulses with few flickers were recorded in the presence of 10 μM of the (+) isomer (19) (Fig. 5). The mean open times were shorter than those induced by ACh, e.g., 5.2 vs. 13 msec, at -140 mV holding potentials. However, the decrease of the mean open time with increasing concentrations of (+) physostigmine and the gradual change in its sensitivity to membrane voltage suggested that at this concentration range this isomer may be acting as an open channel blocker (see below). This pattern was also exhibited by the (-) enantiomer. Therefore, the actual characteristics of neither the (+) nor the (-) physostigmine-activated currents could be determined.

Blocking actions of (+) and (-) physostigmine. The studies of the effects of the physostigmine enantiomers on the nerve-elicited EPCs confirmed the previous report of the lack of stereospecificity of noncompetitive blockade of ion-channel sites (22). With (+) physostigmine, analysis of EPCs eliminated the possibility of any significant anti-AChE activity, as indicated by the absence of the potentiation of peak amplitude and prolongation of EPC decay typical of AChE inhibitors, including the (-) isomer (Fig. 6; see also Fig. 1-5 of ref. 21 and Fig. 5 of ref. 19). Also, the data showed that, similar to the (-) isomer, (+) physostigmine, at concentrations higher than 2 μM, produced significant reduction of both peak amplitude and τ_{EPC}, suggesting noncompetitive blockade of the open AChR-ion channels in a manner described by the sequential model presented earlier (19).

Single channel currents, however, enabled us to distinguish differences in the alterations induced by these enantiomers on the microkinetics of the ACh-activated currents. Similar to recordings obtained with physostigmine enantiomers alone, (-)physo-

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(−) Physostigmine (0.5 μM)

(+) Physostigmine (10 μM)

3 pA

50 msec
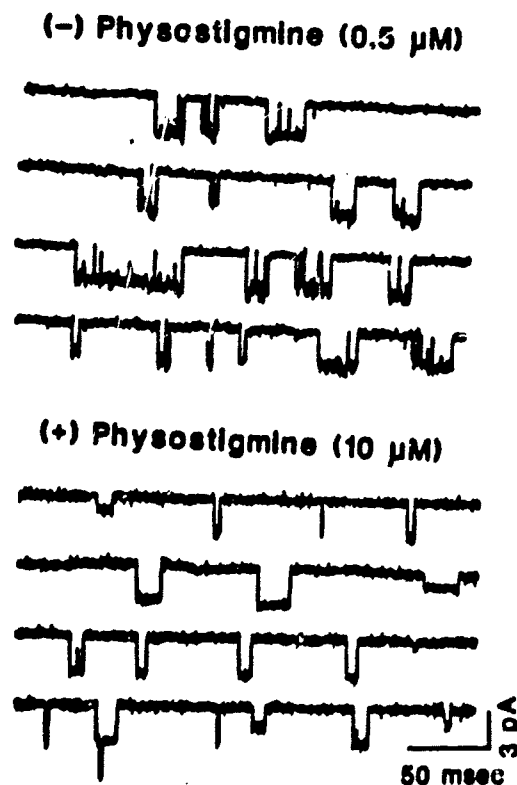
Figure 5. Samples of single-channel currents (cell-attached configuration) activated by isomers of physostigmine. Recorded from frog interosseal muscle at 10°C. Data were filtered at 3 kHz. A similar pattern was observed in all 20 muscle fibers studied.

stigmine produced channel blockade characterized by very fast blocking and unblocking reactions and, therefore, induced burst-like events whereas in the presence of the (+) isomer, the events appeared as well-separated brief square-wave-like pulses. Bursts could not be discerned, denoting a very slow unblocking rate. The blocking actions of (+) physostigmine reflected a decreased mean channel open time (τ_o) that was both concentration and voltage-dependent (Fig. 7). The blockade increased linearly with (+) physostigmine concentration (1-50 μM), and exponentially with hyperpolarization. In addition, as the concentrations of (+) physostigmine increased, the semilogarithmic plots of τ_o vs. membrane holding potentials disclosed a progressive loss of the voltage dependence typical of control ACh-activated currents; at high concentrations (>20 μM) an inversion of the slope sign of these plots was observed.

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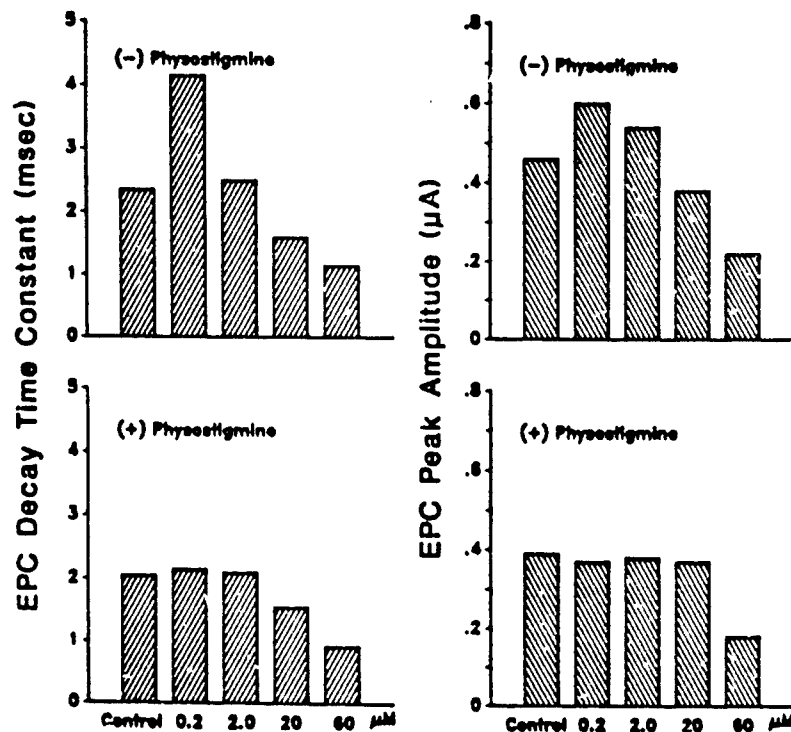
Figure 6. Effect of isomers of physostigmine on the endplate current (EPC) recorded from frog sciatic nerve-sartorius muscle preparation at 21°C. Values given were obtained at -100 mV holding potential. Note that, in contrast to (-) physostigmine, (+) physostigmine produced neither increase in peak amplitude nor lengthening of decay time constant due to the lack of AChE blockade. The data were collected from a total of 12 sartorius muscles. The standard error was within 7% of the mean values in all cases.

The sequential model introduced earlier (23,24) was used to analyze (+) physostigmine actions. According to this model, in the presence of the blocker, the reciprocal of the mean open times ($1/\tau_o$) is governed by the rate constants k_{-2} and k_3, and is linearly dependent on the concentration of the blocker. It can be represented by the following equation: $1/\tau_o = (k_{-2}(V) + k_3(V) \times [D])$. The reversal in the slope of the plot of τ_o vs. membrane potential can be attributed to the strong voltage dependence of k_3, which is opposite to that of k_{-2}.

The lack of clearly defined bursts in the presence of (+) physostigmine precluded the determination of both blocked and burst times. This type of long-lasting blockade was also described for other drugs, such as local anesthetics bupivacaine (25) and QX314 (26) and the OP compound VX (27). Other carbamates -- such as neostigmine, pyridostigmine

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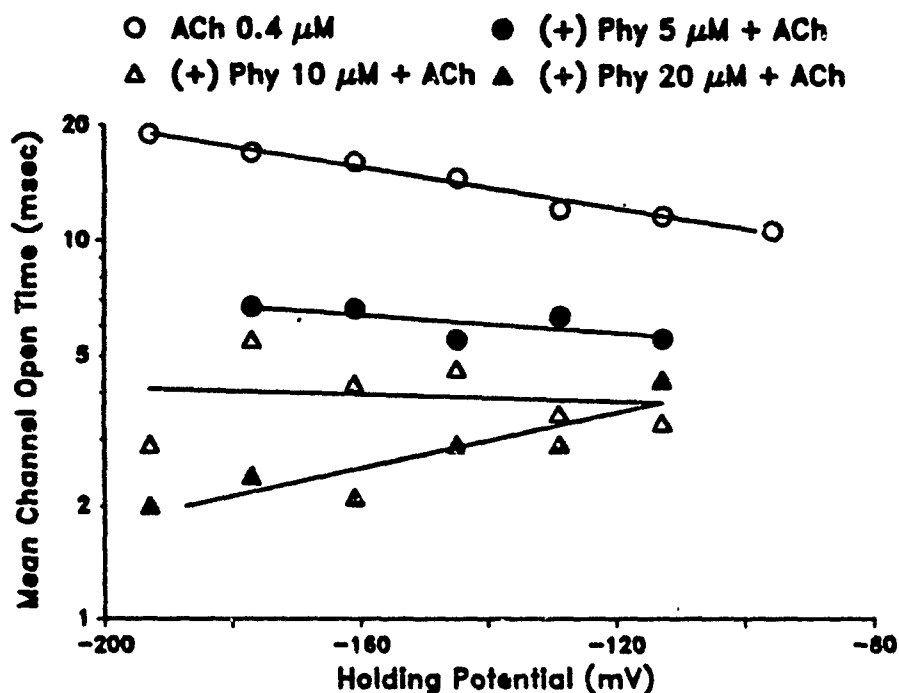
Figure 7. Relationship between mean channel open time and holding potential of channels activated by ACh in the absence and in the presence of different concentrations of (+) physostigmine. Note the marked decrease in mean channel lifetime as a function of (+) physostigmine concentration and membrane potential. Each symbol represents the pooled mean values obtained from 2 to 4 patches whose holding potential ranged ± 2 mV around the mean value mentioned. The entire data for this graph were collected from at least 20 muscle fibers. The standard error was within 8% of the mean value. Solid lines represent the best fit obtained by linear regression.

and edrophonium -- also blocked open nicotinic AChR channels, but with dissociation rates that were intermediate between the two physostigmine enantiomers (19).

Agonist properties and open channel blockade by the carbamates neostigmine and edrophonium.

Although neostigmine and edrophonium at very high concentrations have agonist activity at the neuromuscular AChR, the primary effect of these agents results from their interactions with the open state of ion channels activated by ACh. In the presence of neostigmine and edrophonium at concentrations between 0.2 and 50 μM, channel currents, normally rectangular pulses, were chopped into bursts of rapid openings and closings. Channel open times, i.e., the multiple open intervals within a burst, were shortened in a

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concentration- and voltage-dependent manner. The open time distribution was fit to a single exponential function denoting existence of one open state. The blocking effects were more pronounced at hyperpolarized potentials, and the strong voltage sensitivity of mean channel open times (τ_o), observed under control conditions was progressively decreased by increasing drug concentrations. These alterations were kinetically consistent with the predictions of the sequential model for open channel blockade.

Neostigmine and edrophonium also displayed some agonistic properties, though only at very high concentrations. Neostigmine, at concentrations >20 μM, generated infrequent and very brief channel currents. At high concentrations (e.g., 50-100 μM), some bursts composed of very fast openings and closings were observed. Single channel conductance was similar to that of channels activated by ACh. Edrophonium, on the other hand, activated channel openings with irregular and increased noise levels during the open state; the openings tended to disappear at the hyperpolarized potentials at which recordings were made and reappear after a period of depolarization.

Molecular mechanisms for antidotal efficacy of carbamates.

Electrophysiological, toxicological and morphological studies have provided strong evidence that all the carbamates clinically used as AChE inhibitors -- namely neostigmine, pyridostigmine, edrophonium and (-) physostigmine, interfere with neuromuscular transmission not only by preventing ACh hydrolysis but also by directly interacting with site(s) located on the AChR macromolecule (9,15-19,21). The morphology of the neuromuscular junction and the function of the postsynaptic AChRs were altered differently depending upon the carbamate applied because each of these compounds interacts with multiple targets at nicotinic synapses, producing a particular spectrum of alterations in the kinetics of the AChR activation process. In addition, we believe that, because of these interactions with the AChR macromolecule, some of the carbamates are more effective than others as antidotes against poisoning by a particular OP (13,15-19). Many lines of evidence have also emerged from our studies that have strengthened this hypothesis:

i) (+) Physostigmine, a synthetic isomer of the natural (-) form, in spite of having no significant anti-AChE activity, prevented OP-induced myopathic lesions and protected animals against lethal doses of irreversible AChE inhibitors (see Fig. 1 in ref. 17).

ii) Though with different affinity and potency, all of these carbamates, including (+) physostigmine and the non-carbamate edrophonium, can a) activate or competitively block AChR channels through interactions with ACh recognition sites, and b) alter the kinetics of AChR-ion channel activation through noncompetitive site(s) and produce different types of ion channel blockade, such as reversible open-channel blockade, closed-channel blockade, desensitization, etc. Usually, the final effect results from a combination of two or more of these actions (19).

iii) Carbamates have distinct antidotal potencies and selective actions against OPs. Accordingly, among the carbamates, (+) and (-) physostigmine provided the best protection to animals exposed to lethal doses of OPs (13,15,17,18). In vitro experiments carried out for light microscopic and ultrastructural analyses confirmed these findings. Thus, sublethal

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doses (0.08 mg/kg) of sarin produced a severe and extensive loss of band pattern and induced vacuolation, supercontracture of the subjunctional regions, and phagocyte infiltration, whereas rats pretreated with (-) or (+) physostigmine and injected with lethal doses of sarin (0.13 mg/kg) showed marked reduction in the severity and extent of neuromuscular synapse destruction (Fig. 4; see refs. 17,18).

v) (-) Physostigmine's prophylactic potency was greatly enhanced by co-administration with drugs, such as mecamylamine or chlorisondamine, that had no anti-AChE activity but exhibited definite blocking actions on the nicotinic AChR (14,15).

3. Oximes and related compounds.

Antidotal potency: specificity against OPs. Studies carried out with 2-PAM and HI-6, mono- and bispyridinium oximes, respectively, in phrenic nerve-diaphragm preparations disclosed that in general, HI-6 was more potent than 2-PAM. However, against tabun and soman, a very specific antidotal interaction occurred which was independent of the AChE-reactivation potency (Table 4). Against tabun, in spite of insignificant reactivation of the enzyme (less than 5%), 2-PAM produced complete recovery of twitch and tetanic tension blocked by the OP, whereas HI-6, although reactivating AChE to a higher level (21%) than 2-PAM, was unable to provide any improvement of muscle function. On the other hand, against soman, HI-6 was effective in restoring muscle function, although it also reactivated the same 21% of the AChE activity. 2-PAM, which activated 18% of the AChE was ineffective in recovering tetanus-sustaining ability in muscles exposed to soman. Against VX and sarin poisoning, in spite of better reactivation of AChE activity by HI-6 (100% vs. 50-70% by 2-PAM), both oximes were equally effective in restoring muscle function.

More recent studies carried out with SAD-128, a bispyridinium compound closely related to HI-6, reinforced the hypothesis of a mechanism unrelated to AChE reactivation underlying the antidotal actions of the classical oximes. SAD-128, although devoid of an oxime moiety which confers the AChE-reactivating effect, provided effective protection of animals exposed to lethal doses of soman (28-30). SAD-128 produced alterations in the kinetics of the ion channels activated by the neurotransmitter that were quite similar to those produced by 2-PAM and HI-6; however, SAD-128 was even more potent in producing these effects than were the other two compounds. These alterations resulted from the direct interactions of these compounds with sites located on the ion channel component of the nicotinic AChR (31,32). When the actions were studied in detail at the single channel current level, all the compounds showed definite actions on the nicotinic AChR: they enhanced its activation, blocked the open ion channels, and/or accelerated its recovery from the desensitized state. The differential contribution of all these actions accounted for the relative efficacy and the selectivity of the compounds in relation to a particular OP.

TABLE 4

Effects of 2-PAM and HI-6 on the Recovery of Diaphragm Muscle Function after Depression by OP Exposure

OP and Dose (μM)	Experimental Condition	Twitch Tension (g)	Tetanic Tension at 50 Hz (g)	Tetanus Sustaining Ability	AchE Activity (% Control)
Soman (0.2)	Before exposure	22 ± 3[a]	78 ± 4	100	100
	15 min OP exposure	10 ± 2	10 ± 2	12	4
	HI-6[b]	24 ± 6	53 ± 12	100	21
	2-PAM[b]	13 ± 2	28 ± 3	0	18
Tabun (0.4)	Before exposure	22 ± 3	67 ± 11	100	100
	15 min OP exposure	11 ± 3	11 ± 4	0	6
	HI-6[b]	29 ± 8	41 ± 11	2	21
	2-PAM[b]	22 ± 1	67 ± 9	100	6

[a] Tetanus sustaining ability was measured by recording initial and final tension during a 4 sec tetanus at 50 Hz. Ratio of Final tension/Initial tension X 100 is expressed as tetanus sustaining ability.

[b] Muscles were treated with HI-6 (0.1 mM) or 2-PAM (0.1 mM) for 1 hr after 15 min exposure to OP and subsequent removal of its excess.

[a] Values are means ± S.E.M. recorded from 4 muscles.

Activation and blockade of the post-synaptic nicotinic AChR. 2-PAM and HI-6 did not affect presynaptic elements, membrane electric properties or the contractile apparatus. Thus neither of these compounds affected resting membrane potential, action potential generation or muscle twitches elicited by direct stimulation, and, at high micromolar or even millimolar concentrations, they failed to alter significantly the neurotransmitter release process, as determined by quantal content, quantal size and frequency of spontaneously occurring miniature endplate potentials (MEPPs) (31). Therefore, most of the effects were restricted to motor endplate AChRs.

Increase in AChR activation. This effect was particularly evident with 2-PAM, and resulted from an increase in activation of the post synaptic AChR (Fig. 8; see also Fig. 16
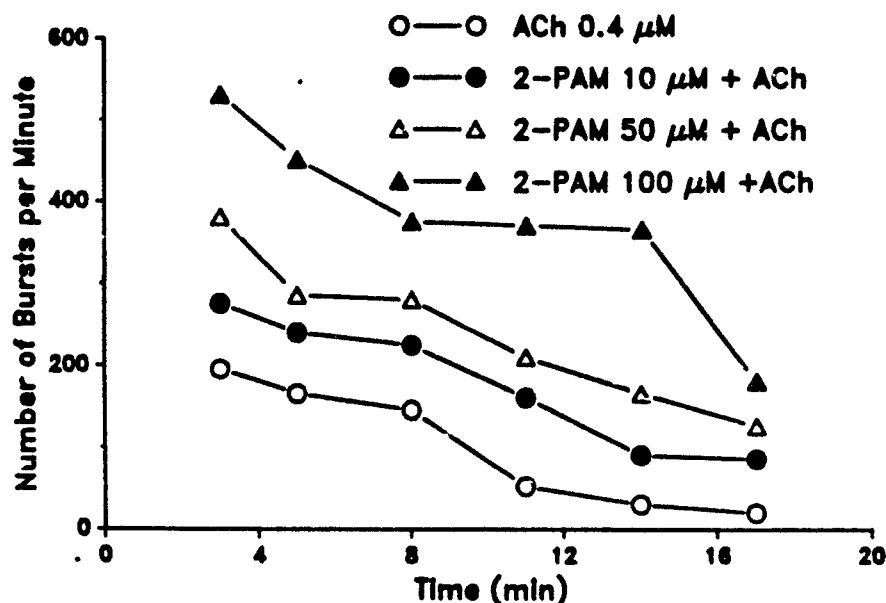
Figure 8. Effect of increasing concentrations of 2-PAM on the frequency of channel activation produced by ACh. This increase in channel opening probability in the presence of 2-PAM could be of significant value in revitalizing the function of OP-poisoned endplates. Each symbol represents the mean number of bursts per minute collected every 3 min (2 min on a few points) after getting the patch seal. Events obtained up to the first min after the seal were not included. The holding potentials at the initial points were -80 mV to -100 mV and the subsequent points along the time scale had a 20 mV increase in hyperpolarization. The data were collected from a total of 10 fibers. The standard error was within 10% of the mean at all data points.

ref. 31) since, as mentioned before, this oxime and others did not affect presynaptic processes (31). Also, AChE inhibition was not sufficient to account for this facilitation, because enzyme activity was affected only at much higher doses of this oxime (31). At the macroscopic level, this effect resulted in potentiation of muscle twitch tension and increased peak amplitude of the EPCs at holding potentials ranging from -50 to +50 mV. As described below, at more negative potentials, blocking actions became so prevalent that the facilitatory effects were not evident.

Single channel recordings provided the ultimate evidence for the direct interactions of these compounds with the AChR sites. Neither 2-PAM, nor HI-6, nor SAD-128 produced channel openings by themselves, i.e., when applied in the patch micropipette alone, without ACh. However, in the presence of ACh (0.4 μM), 2-PAM (Fig. 8) and HI-6 produced a marked concentration-dependent increase in the frequency of bursts. Under control conditions, ACh (0.4 μM) activated channel openings, which appeared as square-wave pulses with very few flickers during the open state and no clear bursting activity. At this concentration, desensitization appeared very slowly, as evidenced by the gradual decline of the frequency of openings over the 40- to 60-min recording period.

Upon the addition of 2-PAM at concentrations of 1-50 μM along with 0.4 μM ACh, the frequency curve was shifted upward while maintaining the same slope of the declining phase (Fig. 8). Although dependent upon oxime concentration, this increase in the frequency was neither voltage- nor time-dependent. With HI-6, this facilitatory effect was significantly less marked, and it was not seen with SAD-128.

The increased channel activation could result from a primary action of 2-PAM increasing the affinity of ACh for its binding site and/or the isomerization rate constant, facilitating the ion channel opening. Another possible mechanism for 2-PAM action is that this oxime could enhance channel activation by counteracting the already existing agonist-mediated receptor desensitization. This explanation is particularly tempting, considering that OPs block neuromuscular transmission mostly by enhancing AChR desensitization, either by ACh accumulation or by direct actions of OPs, or both (31). Indeed, OPs have been reported to enhance AChR desensitization through direct interactions with the nicotinic AChR molecule (33).

Assuming that there is no synthesis or incorporation into the muscle membrane of new nicotinic AChRs during patch clamp recording, one could argue that, in the presence of 2-PAM, more receptors become available for ACh activation. This greater availability of activatable AChRs could result from the shift of the existing AChRs from the desensitized state. It is known that the neurotransmitter and other nicotinic agonists, at equilibrium, shift the AChRs from a low agonist-affinity state to a high agonist-affinity state(s) responsible for the development of desensitization (34). Biochemical and electrophysiological techniques have disclosed at least two phases of desensitization (35,36). The onset of fast desensitization occurring on a millisecond time scale would usually be missed under control patch clamp recording conditions. Therefore, our recordings obtained with ACh alone may depict only the activation of those receptors that escaped the fast desensitization induced by the agonist. Under these conditions, the increased channel activation could result from 2-PAM's ability to prevent AChR isomerization toward a desensitized state. Slow desensitization, however, appeared to be refractory to 2-PAM's facilitatory actions since, at all concentrations of this oxime, parallel decline of channel activation was observed following the initial increase in frequency of openings.

Blockade of AChR-ion channels. The analyses of the kinetics of the macroscopic EPC decays and single channel currents disclosed noncompetitive blockade of the AChR function through direct interactions of 2-PAM, HI-6 and SAD-128 with site(s) on the AChR-ion channels. The ion channel blockade was more evident with HI-6 and SAD-128. On the macroscopic EPCs, plots of τ_{EPC} vs. membrane holding potentials revealed ion channel blockade only at hyperpolarized potentials (from -150 to -80 mV). Denoting a very strongly voltage-dependent process, the decrease of the decay time constant (τ_{EPC}) was accompanied by an inversion of the slope sign of these plots as the concentration of these drugs was increased. Whereas in the presence of HI-6 (1 μM to 2 mM), the acceleration of the EPC decay occurred without changing the single exponential function observed under control conditions, with SAD-128, double exponential decays could be discerned at all concentrations (10-100 μM) tested at membrane potentials between -150 and -100 mV.

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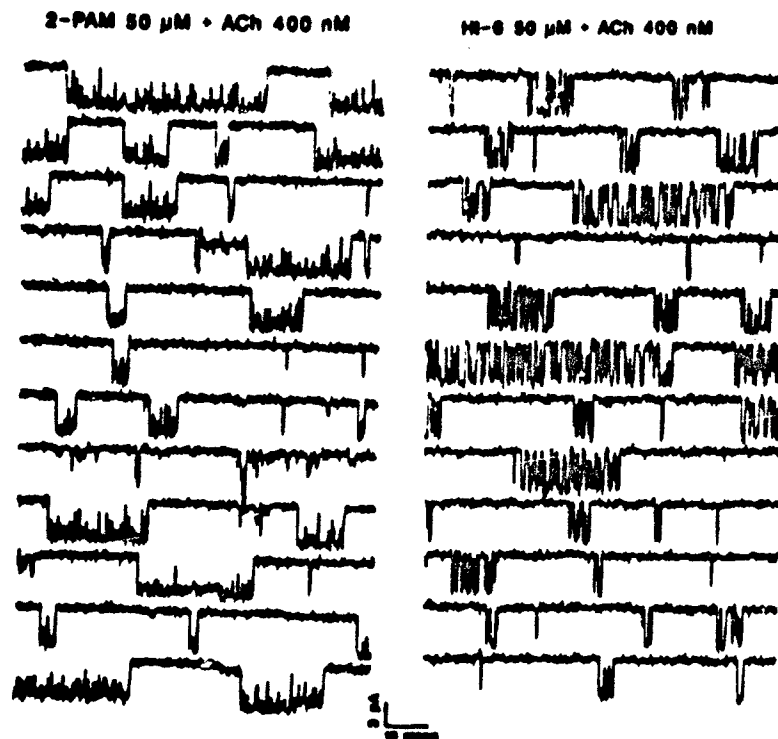
Figure 9. Samples of ACh-activated channel currents recorded from frog interosseal muscle in the presence of 2-PAM (50 μM) (left) or HI-6 (50 μM) (right) included in the patch pipette solution together with ACh (400 nM). Holding potential, -165 mV. A similar pattern was observed in all the 40 muscle fibers studied in this group.

For better interpretation of these alterations, the microkinetics of the elementary currents were analyzed. 2-PAM (10-200 μM), HI-6 (1-50 μM) (Fig. 9) and SAD-128 (1-40 μM) (Fig. 10), when added to fixed concentrations of ACh (0.4 μM for 2-PAM and HI-6, and 0.1-0.2 μM for SAD-128), induced openings with marked increase in the frequency of flickers during the open state as compared to control ACh-induced currents. This flickering was interpreted as resulting from successive blocking and unblocking reactions before the ion channel was closed towards its resting state. The bursts with SAD-128 were much longer than those observed in the presence of 2-PAM or HI-6 because of the much longer-lasting blocked states. With HI-6 and especially with 2-PAM, the high frequency of these flickers made the noise level during the open state broader than that observed during the closed state or in the absence of channel activity. In addition, as the frequency of these flickers increased with higher concentrations of these oximes (>100 μM), the inadequate recording and digitization of the very fast events resulted in an apparent decrease in the single channel conductance.

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TABLE 5

Comparison of the Channel-Blocking Rates[a] for Different Pyridinium Drugs

Holding Potential (mV)	$k_3 \times 10^{-6} \sec^{-1} M^{-1}$		
	2-PAM	HI-6	SAD-128
-100	2.7	8.7	104
-120	4.0	14.5	130
-140	5.9	24.0	148
-160	8.7	39.6	170

[a] The blocking rates were obtained from single channel studies from frog muscle fibers. Blocking rates were calculated from the slope of the linear regression plot of drug concentration vs. reciprocal of mean open time. The open time data were collected from more than 50 muscle fiber patches for the three pyridinium drugs used.

The analysis of the open-channel kinetics showed that both the oximes 2-PAM and HI-6 and the compound SAD-128 produced a concentration- and voltage-dependent reduction of the mean open times (Fig. 11). At low concentrations, this effect was apparent only at very hyperpolarized potentials. As the concentration of these agents increased, the effect became apparent at less negative potentials. The voltage dependence of the mean open times followed the predictions of the sequential model used to describe the actions of many ion channel blockers and presented before. As discussed earlier, the opposite voltage dependence of the rate constants k_{-2} and k_3 resulted in the blocking pattern exhibited by these drugs. Table 5 shows the k_3 ($\sec^{-1}.M^{-1}$) values and voltage sensitivity for 2-PAM, HI-6 and SAD-128. The k_3 values changed an e-fold per 52 mV and 40 mV for 2-PAM and HI-6. SAD-128 blocking actions were less voltage dependent; k_3 for this drug changed an e-fold per 150 mV. Also, many other blockers, such as QX-222 and (-) physostigmine, produced a much less voltage-dependent reduction of the mean open times.

Analysis of the distributions of the closed times showed that in the presence of these drugs, they were best fitted by the sum of two exponentials. The fast component represented the numerous fast flickers or blocked states induced by 2-PAM and HI-6 and, on a much slower time scale, by SAD-128. With both oximes, 2-PAM and HI-6, the two components in the closed-time histograms could be easily discriminated. However, due to the slow transitions between the blocked and open states in the presence of SAD-128, the fast component could be adequately separated only in recordings with very low frequency

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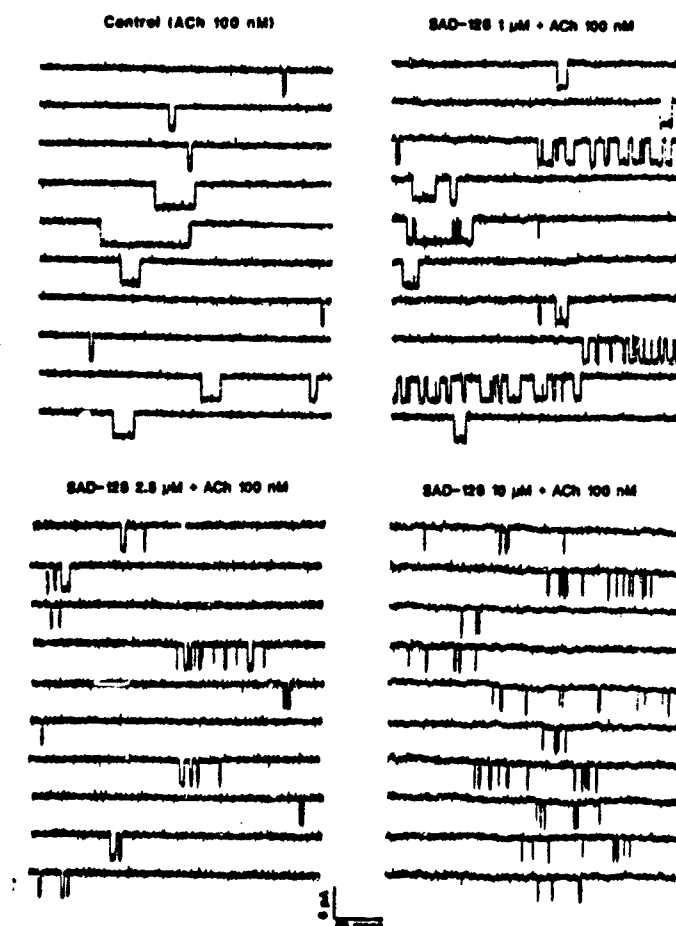
Figure 10.   Samples of ACh-activated single-channel currents recorded from frog interosseal muscle in the absence and presence of SAD-128 inside the patch pipette solution.   Holding potential, -140 mV.   A similar pattern was observed in all the 25 muscle fibers studied in this group.

of channel openings, which permitted a good burst discrimination.   For 2-PAM, the fit of the fast distribution to a single exponential function provided a mean of about 130 μsec at all potentials where the blockade appeared.   For HI-6, this mean was voltage dependent such that the values were 140 μsec and 390 μsec at holding potentials of -120 mV and -180 mV, respectively.   The mean blocked times for SAD-128 were also voltage dependent, but were 10- to 20-fold longer than those for HI-6.   According to the sequential model, the mean blocked times depend solely on the rate constant for the unblocking reaction (k_{-3}).   The values and the voltage dependence of k_{-3} determined from the recipro-

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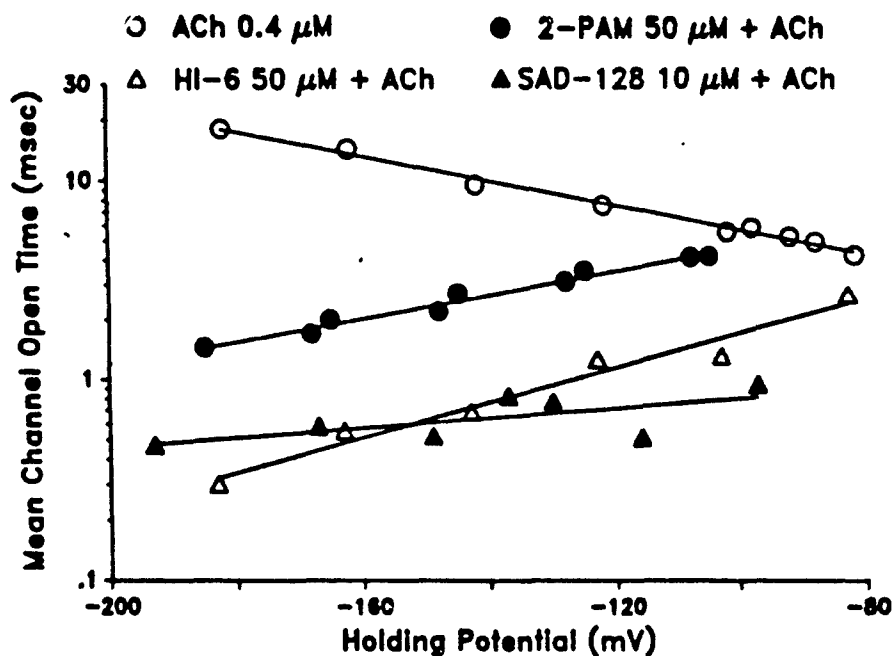
Figure 11. Relationship between mean channel open time and holding potential of channels activated by ACh in the absence and in the presence of 2-PAM, HI-6 or SAD-128. Each symbol represents the mean fit value obtained from 1000 to 5000 events in each and the standard error for each symbol was within 5% of the mean values. Solid lines represent the best fit obtained by linear regression. The data were collected from a total of 15 muscle fibers.

reciprocals of the mean blocked time are shown in Table 6. The k_{-3} values changed an e-fold per 58 and 32 mV for HI-6 and SAD-128, respectively, whereas the dissociation rate constant for 2-PAM was not significantly influenced by the voltage.

The dissociation constant (K_D) values obtained for 2-PAM and HI-6 were 1.27 and 0.204 mM, respectively, at -140 mV holding potential. In comparison to HI-6, SAD-128's K_D value was almost 100- to 150-fold lower, around 1.5 μM at -140 mV holding potential. The high K_D values for the oximes indicated that they bind to a low-affinity site and that SAD-128 without oxime function, is more potent in blocking ACh-activated channels. Using the Boltzmann distribution to describe the voltage dependence of K_D values, the location of the binding site can be estimated. For both 2-PAM and HI-6, the binding site is roughly halfway through the membrane (31). Similar values were determined for SAD-128 (32) and for other blockers, such as neostigmine and edrophonium (19), suggesting that they bind to the same site with different affinities.

Some additional features of the blockade produced by these drugs can be discussed in light of the predictions of the sequential model. The following points argue in favor of the sequential blocking model: i) a linear decrease in the mean open times with concentration of the blocker (for 2-PAM up to 100 μM and for HI-6, up to 50 μM); ii) an

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TABLE 6

Comparison of the Channel-Unblocking Rates[a] for Different Pyridinium Drugs

Holding Potential (mV)	$k_{-3} \times 10^{-3}$ sec^{-1}		
	2-PAM	HI-6	SAD-128
-100	7.8	9.9	0.70
-120	7.8	7.1	0.38
-140	7.8	5.0	0.21
-160	7.8	3.6	0.12

[a] All the unblocking rates were obtained from single channel studies from frog muscle fibers. The rates were derived by reciprocating the mean values of the blocked times. The blocked times were collected from more than 50 muscle fiber patches for the three pyridinium drugs used.

increase in the mean blocked time with hyperpolarization in the case of HI-6 and SAD-128, but not of 2-PAM; and iii) the blocked times independent of drug concentration (for HI-6, up to 50 μM and for SAD-128, up to 40 μM). However, some deviations from the predictions of the sequential model have been observed with the oximes and with SAD-128 that can be enumerated as follows: The model predicts that i) the total time that the channel spends in the open state is unaltered by the blocker, and ii) both the number of flickers or openings per burst and the mean burst time should increase with drug concentration. The analysis showed that the total open time in a burst and also the duration of the bursts were decreased in a voltage-dependent manner as the concentrations of these drugs increased. The number of openings per burst increased with concentration, but at higher doses of the oximes (particularly with HI-6) and at very negative holding potentials, a reduction in this number was observed. With a typical blocker like QX-222 (up to 40 μM), the mean burst time, along with the number of openings per burst, increased with concentration, thus maintaining the total open time per burst equal to that determined in the absence of the drug (26). iii) With SAD-128, the linear relationship between the reciprocal of τ_o and its concentration predicted by the model was only observed at a low concentration of the blocker (up to 10 μM). Above this concentration, a departure from the linearity became evident (32).

These deviations from the simple sequential blocking mechanism require the existence of alternate routes for oxime and SAD-128 actions on the nicotinic AChR. One could suggest several possibilities: i) A new stable blocked or desensitized state can be reached either directly from the open (A_2R^*) or from the previously existing blocking state (A_2R^*D). ii) There occurs an isomerization of A_2R^*D directly to the closed or resting state, bypassing the open state (A_2R^*), thereby reducing the total open times per burst

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and the mean burst length. The dissociation of one or two agonist molecules could force an isomerization towards the closed state. iii) The dissociation of one or two agonist molecules could also force an alteration of the closing rate constant (k_{-2}). These possibilities, although not proved from our data, are probable.

AChE-like actions of the oximes. It has been reported that hydroxylamine is able to hydrolyze acetylcholine, in a manner similar to the action of the enzyme AChE (37). Therefore, the existence of a similar reaction between either 2-PAM or HI-6 and acetylthiocholine used as substrate was investigated. The data in Table 7 indicate significant hydrolysis, particularly with 2-PAM, which was about 2-2.5 times more potent than HI-6. This reaction could also be predicted to occur between these oximes and the neurotransmitter ACh. Although such a hydrolysis plays no role under normal conditions (with AChE fully functioning), it may be of great relevance under conditions of irreversible phosphorylation of the enzyme, since hydrolysis would reduce the activity of excess ACh in the cholinergic synaptic cleft. The occurrence of this reaction in vivo could partly account for the antidotal efficacy of oximes against OPs. Weak anti-AChE activity observed with high concentrations of 2-PAM may not be of any importance during OP poisoning.

TABLE 7

Interaction Between Pyridinium Drugs and Acetylthiocholine[a]

Concentration of Pyridinium Drug (μM)	Rate of Acetylthiocholine Breakdown (μM/min)		
	2-PAM	HI-6	SAD-128
50	1.1	0.4	0
100	2.0	0.9	0
200	4.8	1.9	0
500	9.7	5.2	0

[a] Values are the means of triplicate samples. Reactions were carried out according to the colorimetric method described by Ellman (38), except that there is no addition of AChE enzyme from any source. A mixture of acetylthiocholine (ATC) (750 μM) 0.1 M phosphate buffer (pH 8.0), dithiobisnitrobenzoate and the pyridinium compound were placed in a 1 ml cuvette and the changes in the optical density was monitored at room temperature continuously for 12 min at 412 nm in an UV spectrophotometer. The rate of ATC breakdown was calculated from the extinction coefficient of the thiocholine, which is released in the reaction between the oximes and ATC.

Molecular mechanisms of the antidotal efficacy of oximes. The oximes 2-PAM and HI-6 and the related compound SAD-128 produce multiple alterations of the AChR function through mechanisms unrelated to reactivation of the phosphorylated AChE. Although these compounds interact with the same sites, the final action of each compound represents the result of distinct contributions of multiple interactions with the nicotinic AChR and of the chemical reaction between the oximes and the neurotransmitter. This differential contribution of the various interactions makes each compound specifically or particularly potent against a given OP (39).

As mentioned before, in the increase of AChR activation, 2-PAM was much more potent than HI-6, whereas SAD-128 was not observed to induce any increase. The increase in the channel opening probability, that as suggested from the data could result from the ability of oximes to arrest fast desensitization, and thus to become relevant under conditions of OP poisoning, when AChR desensitization may be the dominant effect produced not only by excess ACh but also by direct interactions of OP with the nicotinic AChR (40). Thus, 2-PAM, in particular, could counteract the effects of OPs and restore neuromuscular transmission through this mechanism.

In addition, all three compounds produced reversible channel blockade. Comparatively, SAD-128 produced more stable blockade at much lower doses. The availability of activatable AChR through the first mechanism described above, followed by a reversible channel blockade, may release a significant number of AChR from the desensitized state and thereby reestablish the synaptic function.

Finally, an AChE-like action, particularly by 2-PAM and HI-6, may play a significant role in the antidotal efficacy of these agents. This effect, although irrelevant under normal conditions, may greatly contribute to diminishing ACh concentration at the cholinergic synapses in OP-poisoned animals.

4. Patch Clamp Studies on Cells from the Central Nervous System.

Our studies with peripheral nicotinic receptors have shown that apart from AChE inhibition, OPs and prophylactic drugs such as the (-) and (+) isomers of the carbamate physostigmine have direct effects on the AChR. In view of the homology between receptors, peripheral and CNS, interaction with central nicotinic receptors through mechanisms similar to those proposed for peripheral muscle AChRs should play an important role in toxicity of OPs. We have endeavored to characterize the central nicotinic AChR through the use of the single channel recording method and to study its sensitivity to various known nicotinic agonists and antagonists.

Nicotinic AChRs are present at various levels in the nervous system. While receptors located at muscles, ganglia and the brain respond to ACh, pharmacological differences have led to the subclassification of nicotinic AChRs. Peripherally potent AChR ligands bind to two pharmacologically distinct sites in brain tissues, which are also distributed differently in brain regions. The antagonist α-bungarotoxin (α-BGT) binds with high affinity, but has often failed to antagonize nicotinic responses (for review, see ref. 41).

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The second population of AChR is labelled (-)[^{3}H] nicotine and [^{3}H]ACh. Whereas the functional significance of the α-BGT binding site has not yet been defined, a consensus is that the agonist site labelled by [^{3}H]ACh and (-)[^{3}H] nicotine is responsible for some nicotinic cholinergic responses.

The disclosure of functional AChRs in the CNS has been the most difficult endeavor. Biochemical studies showed that (-) nicotine facilitates the release of neurotransmitters from synaptosomes, although this action was insensitive to α-BGT (42). Perhydrohistrionicotoxin, an ion channel probe for the peripheral AChRs, also blocked nicotine-induced transmitter release in mammalian brain. This is evidence for homology of the ion channels of neuronal and muscular AChR (43). Because nicotinic cholinergic pathways have been reported in the hippocampus and the brain-stem reticular formation, cells from these regions in fetal rats were isolated and cultured. Since the central nervous system studies represent a new effort, we are including a brief description of the methods.

(+) Anatoxin-a ((+)Antx): (+)Antx is a naturally occurring semirigid alkaloid produced by the blue-green algae Anabaena flos-aquae. This toxin causes rapid death by activating the peripheral AChR to produce a depolarizing type of neuromuscular paralysis.

Although both ACh and (+)Antx were used as agonists, the nicotinic ligand (+)Antx has several important advantages over ACh:

i).   (+)Antx is not inactivated by AChE,
ii).   (+)Antx is a potent nicotinic agonist with a high degree of stereoselectivity,
iii). (+)Antx is nearly devoid of muscarinic activity,
iv).   At effective agonist concentrations, (+)Antx lacks the noncompetitive blocking effects of (-) nicotine.

Hippocampal neurons cultured for 15 days. Hippocampal cultures contained mostly pyramidal cells, because granule cells are not present at the prenatal stage in these animals. The cells in the brainstem culture were either pyramidal, fusiform or spherical, as described in a recent morphological and electrophysiological study of guinea pig brainstem neurons (44). We found that AChR activity was likely to occur near the base of a dendrite. Thus, most of our patch clamp recordings were obtained from this region (Fig. 12).

Activation of AChR on neurons. Typical single channel currents activated by ACh and (+)Antx (1 μM each) in hippocampal cells are shown in Figure 13. In some patches, the frequency of openings was high enough that currents resulting from the simultaneous openings of two or more channels with similar or different conductance states could be recorded. A concentration of (+)Antx 10-fold higher (0.2-1 μM) than that necessary to activate muscle AChRs (0.02-0.2 μM) was used to induce openings of channels in neurons.

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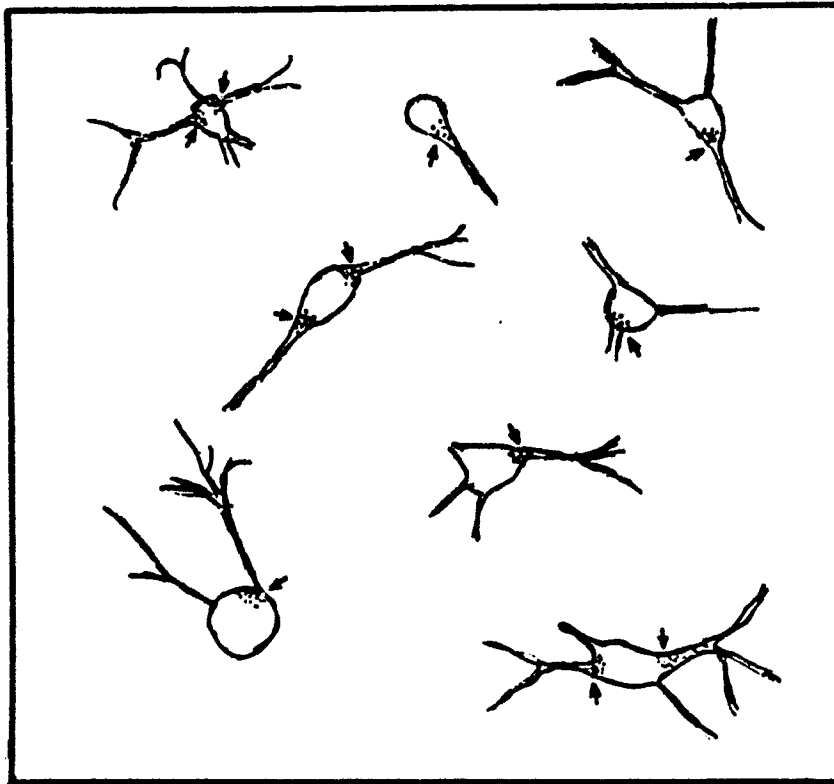
Figure 12. Schematic illustration of the types of hippocampal neurons cultured for 15 days. Picture was handdrawn by viewing the neuron under a high magnification inverted microscope. The indicated dots and arrows were based upon the results of patch clamp recordings. Hippocampal cultures contained mostly pyramidal cells, because granule cells are not present at the prenatal stage of these animals. The cells in the brainstem culture were either pyramidal, fusiform or spherical. We found that AChR activity was likely to occur near the axon hillock (indicated by arrows). Thus, most of our patch clamp recordings were obtained from this region.

Conductance of the neuronal AChR. The predominant population of single channel currents recorded from hippocampal cells showed a single channel conductance of about 20 pS at 10°C, as determined from the slope of the current-voltage relationship (11). Currents activated by both agonists were further analyzed in a subsequent series of experiments carried out at room temperature (22-23°C) using the outside-out patch configuration. Under these conditions it was possible to record both inward and outward currents, and a slope conductance value of 40-45 pS was obtained for the predominant population of single channel currents (Fig. 14). Considering the Q_{10} value of 1.3-1.5, these currents appear to be closer to the 30 pS population than to the dominant 20 pS currents recorded using the cell-attached configuration. The conductance was the same for (+)Antx-activated currents in hippocampal neurons and in retinal ganglion cells (Fig. 14).

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ACh 1 μM   **(+) Anatoxin-a 1 μM**

10 pA
60 msec.
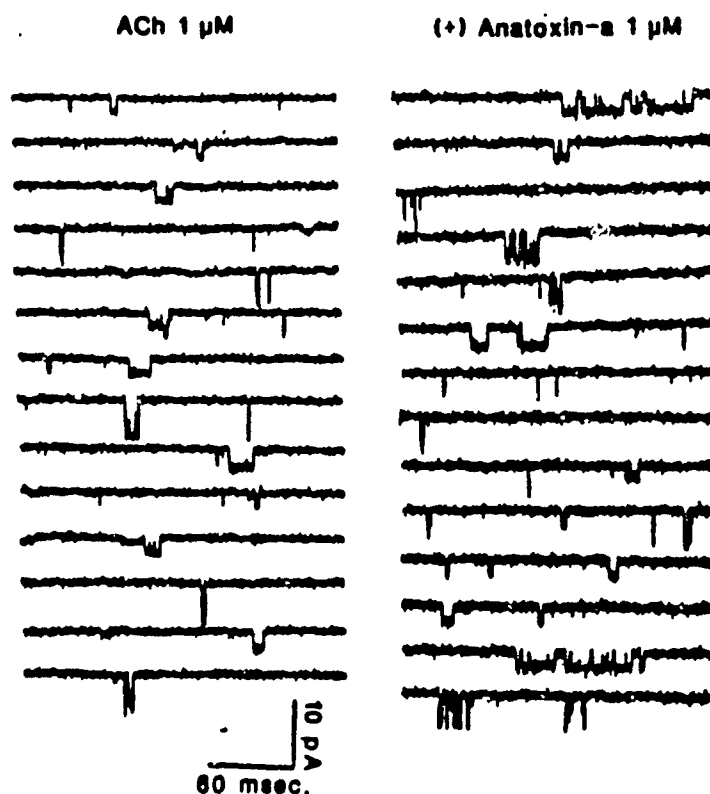
Figure 13. Samples of single channel currents activated by ACh and (+) anatoxin-a recorded from rat fetal cultured hippocampal neurons at room temperature, using cell-attached configuration. Multiple conductance states, typical of immature tissue, are apparent. A similar pattern was observed in at least 5 neurons out of about 100 neurons studied. The rest of the neurons did not respond to these agonists even up to 10 times the concentrations mentioned. Holding potential for ACh was -95 mV and -90 mV for ACh and (+) anatoxin-a groups, respectively.

Kinetic analysis of ion channel activation on neurons. For the kinetic analysis in our first series of experiments using cell-attached configuration at 10°C, the predominant population of 20 pS conductance currents were used. Whereas the ACh-activated currents showed only a few interruptions during the open state of the channels (Fig. 13), the (+)Antx-induced channel openings contained many flickers, giving rise to a double exponential distribution of the closed times. Later experiments carried out at room temperature under outside-out patch clamp configuration revealed that the 40-45 pS currents in hippocampal neurons had a mean open time of about 2 msec at -80 mV and a mean flicker duration of approximately 1 msec. These results are very similar to those obtained in retinal ganglion cells (see histograms shown in Fig. 15). In both types of cells, the number of openings per burst was voltage dependent, the number of flickers increasing with hyperpolarization (Fig. 16).

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Retinal Ganglion Cells   **Hippocampal Neurons**
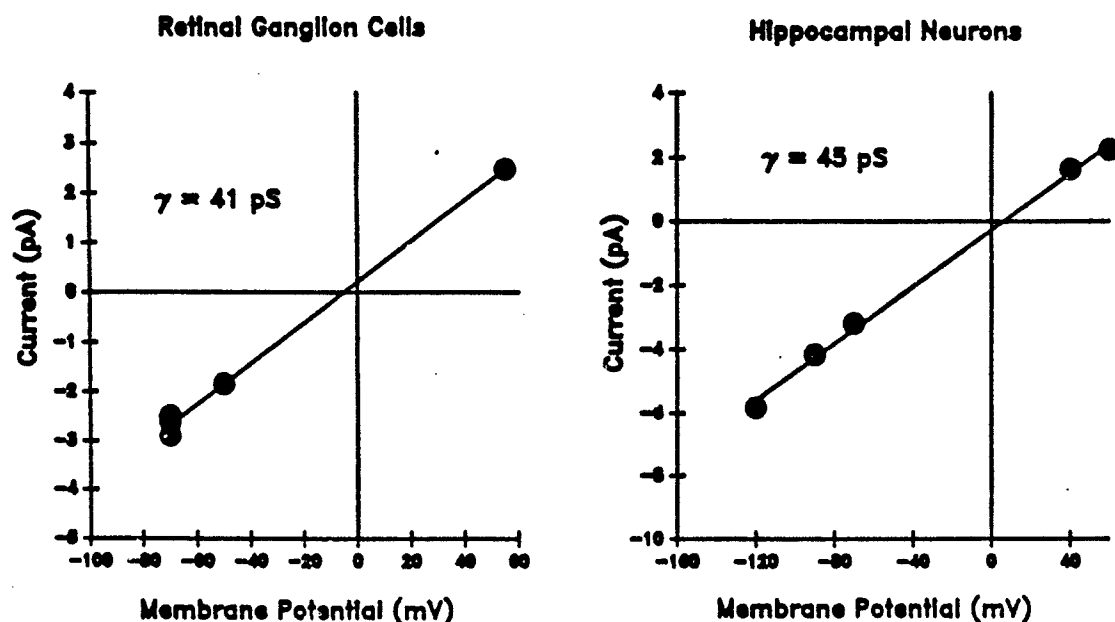

Figure 14. Current-voltage relationship and slope conductance for channels activated by
(+) anatoxin-a (1 μM) in rat cultured ganglion cells and hippocampal neurons.
Recording was made at room temperature using an outside-out configuration. The
data represented are from a single neuron in each group, however, similar values
were obtained in at least 20 or more neurons.

In summary, single channel recording techniques have been applied to neurons
cultured from the hippocampus of fetal rats in order to search for AChRs in the CNS.
In addition to ACh, the potent and specific agonist (+)Antx was used to characterize
nicotinic channels. AChRs were concentrated on the somal surface near the base of the
apical dendrite, and in some patches their density was sufficient to allow the recording of
two or more channel openings simultaneously. Although a multiplicity of conductance
states was also evident, the predominant population showed a single channel conductance
of 20 pS at 10°C. Thus, these neuronal AChRs resembled the embryonic or denervated-
type AChRs in muscle. However, channel opening and closing kinetics were faster than
reported for similar conductance channels in muscle. Therefore, the nicotinic channels
described here are similar but not identical to those of the well-characterized muscle
AChRs, in agreement with biochemical, pharmacological, and molecular genetic studies on
brain AChRs. The typical location of channels at the base of the dendrite suggests an
important regulatory function in integration of synaptic input. To what extent these
nicotinic targets are important in central effects of prophylactic and therapeutic drugs in
counteracting toxicity will be extensively investigated in this laboratory.

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$\tau = 3.0$ msec

$\tau_1 = 0.3$ msec
$\tau_2 = 1.5$ msec

$\tau_1 = 6.5$ msec
$\tau_2 = 52.1$ msec

Channel Open Time (msec)

Channel Closed Time (msec)

Channel Burst Time (msec)
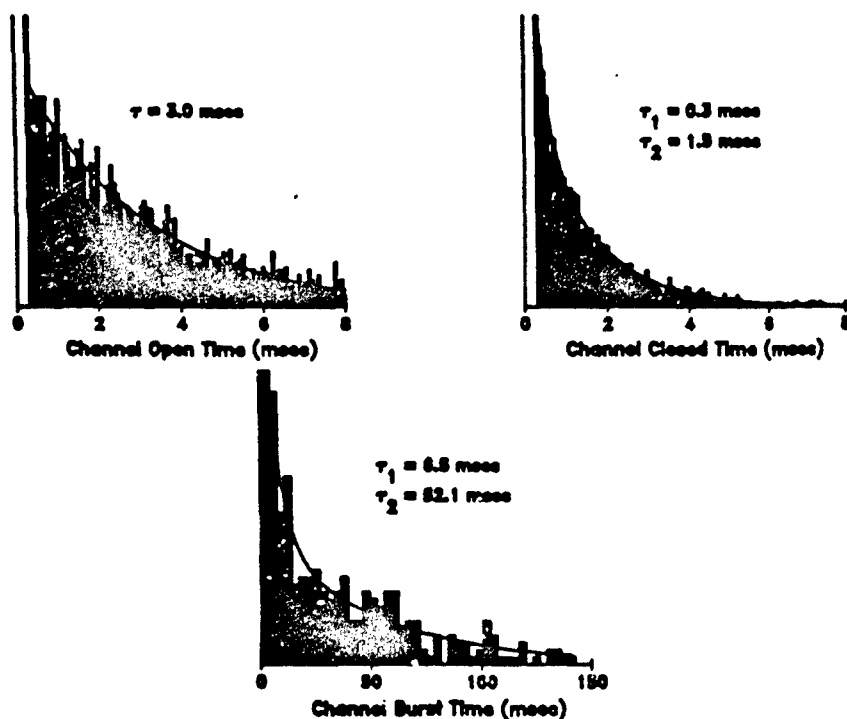
Figure 15. Histograms representing open, closed and burst durations of channels activated by (+) anatoxin-a in rat cultured retinal ganglion cells. Recording was made at room temperature using an outside-out configuration at a holding potential of -65 mV. The curve in each histogram represents the best fit to the data points obtained by nonlinear regression. The data for the histogram were collected from a single experiment. Similar values were obtained in at least 10 or more neurons.

5.   Study of the (+) and (-) enantiomers of nicotine.

At the peripheral nicotinic AChR, nicotine itself has profound effects. Initial studies of Barlow and Hamilton (45) have demonstrated that (+) and (-) nicotine isomers show stereospecificity at the peripheral sites. However, the mechanisms of action of these isomers on the skeletal muscle receptor have not been thoroughly investigated. It is anticipated that several isomers of nicotine will reveal important actions on the central nicotinic AChR, but before studying these agonists' actions on the CNS, we have examined their actions on the frog skeletal muscle in detail.

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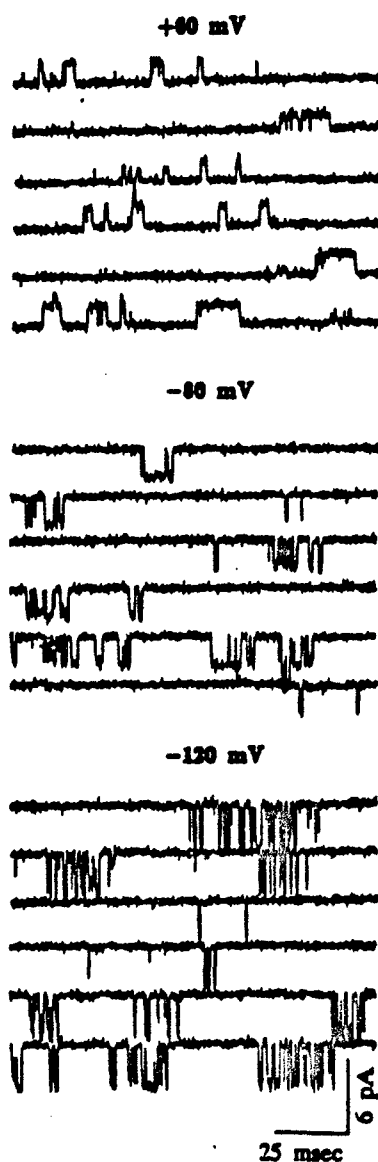
Figure 16. Samples of single channel currents recorded from rat fetal cultured hippocampal neurons in the presence of (+) anatoxin-a (1 μM). Recording was made at room temperature in an outside-out patch at different holding potentials, and the data were filtered at 2 kHz. A similar pattern of activation was obtained in about 7 to 8% of a total of 80 neurons studied. The rest of the neurons did not respond to this agonist even at 10 μM.

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Potency assay of nicotine stereoisomers with rectus abdominis contracture. By direct comparison, the contracture potencies were similar for carbachol and the natural isomer, (-) nicotine. In contrast, synthetic (+) nicotine was 8 times less potent than (-) nicotine. These results are comparable to those previously demonstrating a stereospecific difference in potency of the isomers (45).

Endplate depolarization induced by nicotine stereoisomers. Intracellular microelectrode recordings of single endplate regions of sartorius muscles demonstrated the time course and magnitude of depolarization due to (-) and (+) nicotine (see Fig. 17). After the first hour (control = 100%), the drug was superfused continuously (time 0-60 min) and then washed. In equimolar concentrations (20 μM), (-) nicotine produced a maximal 30% depolarization, whereas (+) nicotine caused only 10% depolarization. Using equipotent concentrations of (-) and (+) nicotine, the magnitude and time course of depolarization were very similar. In all cases the effects at 1 hr became very similar; thus the transient effects were concentration-related, but the final effects were much less sensitive to concentration. Upon washing, the membrane potential recovered completely.

Inhibition of indirect twitch of frog sartorius muscle by nicotine enantiomers. Application of low concentrations, 10 μM (-) nicotine or 20-40 μM (+) nicotine, via the bath initially caused a gradual decrease in twitch tension over 30 min although blockade was incomplete. At higher concentrations, an increase in resting tension occurred within the first minute. Thereafter, both the resting tension and the twitch strength declined until blockade of twitch was achieved in less than 10 min. Washing with drug-free Ringer's solution resulted in a partial recovery of twitch strength.

(-) Nicotine was more potent than (+) nicotine at inhibiting the indirect twitch; a 4-fold greater concentration of (+) nicotine (80 μM) than of (-) nicotine (20 μM) was necessary to achieve complete block in 30 min. The time course, by which (+) nicotine blocked twitch, appeared to be longer than that for (-) nicotine.

Effects of nicotine on repetitive stimulation. After 30 min of treatment, both (-) and (+) nicotine produced a concentration-dependent depression of the EPC amplitude (Fig. 18). However, after repetitive stimulation at 25 Hz for 2 sec, the 50th EPC (-100 mV) was not different from the initial response (average of 6th to 15th responses).

Desensitization. The clustered pattern of channel activity at high concentration (Fig. 19) was typical of desensitizing agonist concentrations.

Endplate current responses during treatment with nicotine. Amplitude and decay time constant were measured during the onset of blockade. Both (-) and (+) nicotine produced very similar results (Fig. 20). When the peak amplitude was depressed 50%, the decay time constant was also slightly reduced. The tendency for τ to be decreased more at hyperpolarized potentials, as well as the curvature in the current-voltage

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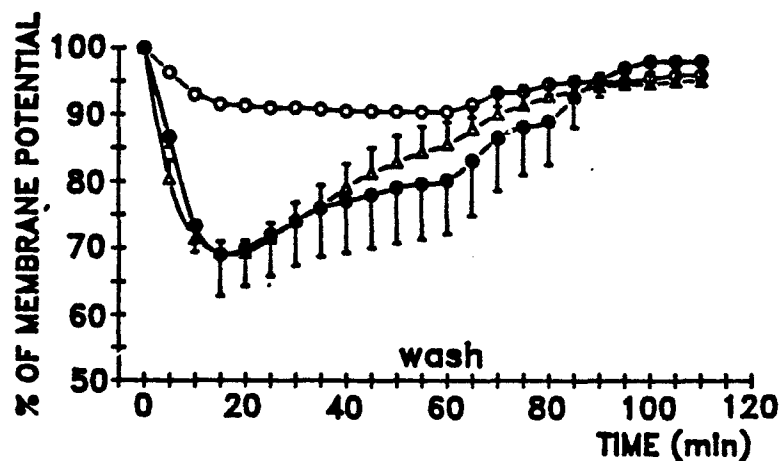
Figure 17. Time course of frog sartorius muscle endplate depolarization and repolarization induced by treatment with 20 μM (-) nicotine (open triangles), or 20 μM (+) nicotine (open circles) or 200 μM (+) nicotine (filled circles). Each point and bar represents the mean membrane potential $\pm$ SE from 3-4 sartorius muscles.
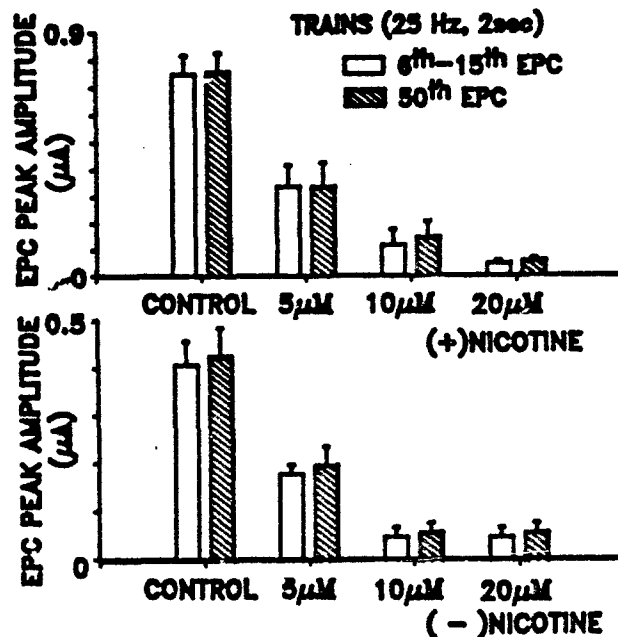
Figure 18. Effects of (-) and (+) nicotine on EPCs elicited by repetitive stimulation. Tetanic stimulations (25 Hz for 2 sec) were applied in control conditions and in the presence of either (+) or (-) nicotine. The amplitude of the last EPC (50th) (striped) was compared with the average of the 6th to 15th EPCs (blank). The membrane potential was kept at -100 mV. Each histogram represents the mean $\pm$ S.E. of EPC peak amplitudes obtained from 4 muscles.

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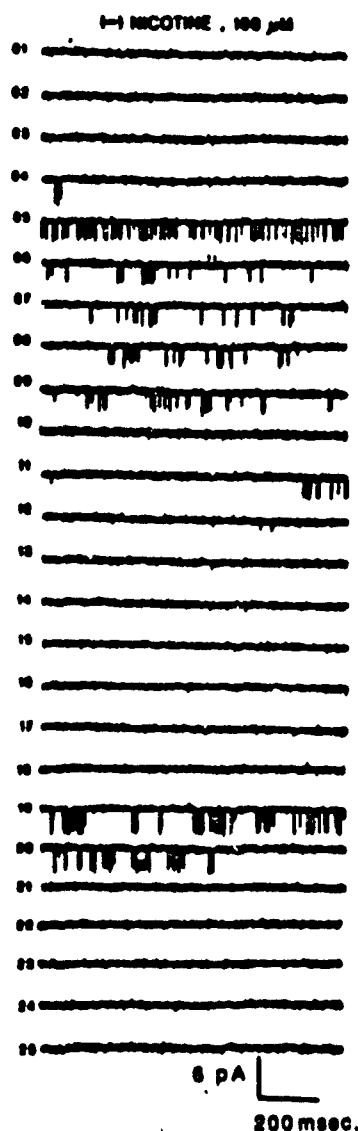
Figure 19. Desensitization in the presence of (-) nicotine. Single channel currents (continuous recording were elicited by (-) nicotine alone in the pipette, cell-attached configuration, 10°C, from a single frog interosseal muscle fiber. A similar pattern was observed in a total of 10 muscle fibers studied.

relationship are suggestive of a noncompetitive ion channel blocking mechanism. However, the magnitude of amplitude depression by far exceeded that which could be produced by this small decrease in τ. Therefore, other mechanisms must contribute. Because nicotine also produces postsynaptic membrane depolarization by stimulation of ACh receptors, competitive agonist effects desynchronized from the evoked transmitter release or desensitization of the receptor are possible.

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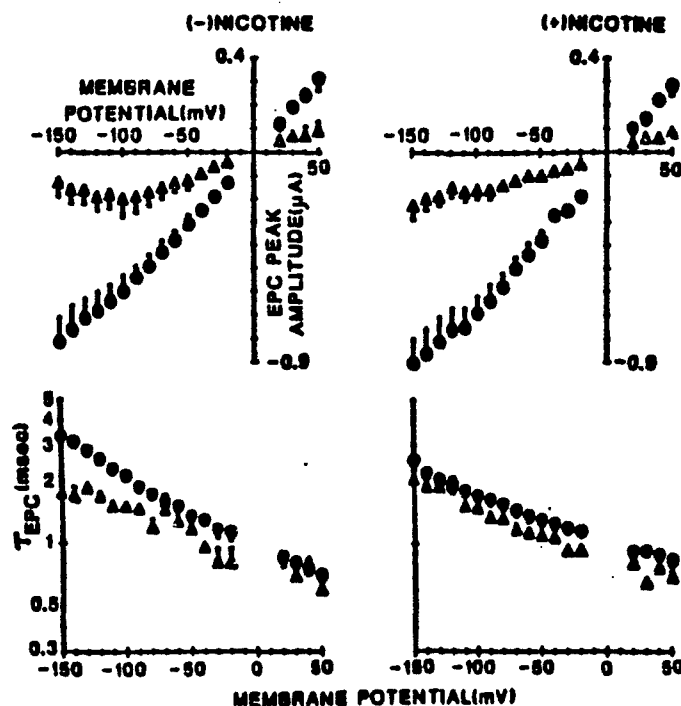
Figure 20. Effects of (-) nicotine (left) and (+) nicotine (right) on peak amplitude and time constant of EPC decay (τ_{EPC}). Upper records depict the relationship between the EPC peak amplitude and the membrane potential under control conditions (circles) and in the presence of 20 μM (-) and (+) nicotine (triangles). Lower records illustrate the relationship between the logarithm of τ_{EPC} and membrane potential under control condition (circles) and in the presence of 20 μM (-) and (+) nicotine (triangles). Each point represents the mean $\pm$ SE from 2-3 sartorius muscles.

Single channel activation by ACh, (-) nicotine and (+) nicotine. When both the nature and concentration of the agonist must be controlled and microscopic kinetics are important, the patch clamp technique is particularly useful. Isolation of the muscle fibers (using a collagenase and protease digestion) removes the nerve terminal and the AChE, leaving only the postsynaptic portion of the endplate. Typical single channel recordings are shown in Fig. 21. In these studies, AChRs were activated by addition of agonist inside the patch pipet and formation of cell-attached patches. The conductance and the kinetic properties were determined for single channels activated by ACh, (-) nicotine, (+) nicotine, or combinations of ACh with each of the nicotine isomers.

Kinetics of nicotinic activation of AChR. With the addition of from 1-10 μM (-) or (+) nicotine, the duration of channel opening was similar to, although in both cases shorter than, that of ACh. Channel openings were separated by brief closures, whose durations (0.1 to 0.2 msec) did not have a clear voltage dependence. This resulted in a decrease in the open time/burst time ratio. There was a slight decrease in the open time

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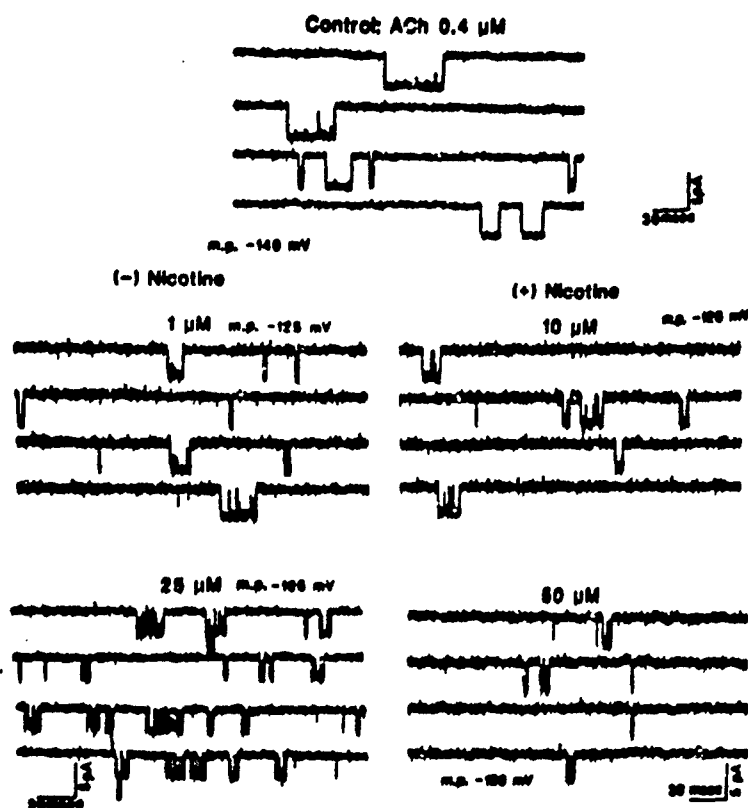
Figure 21. Samples of single channel recordings from frog interosseal muscle fibers under cell-attached conditions, activated by ACh, (-) nicotine and (+) nicotine.

between 1 and 10 μM (-) nicotine. For (+) nicotine, the open time was even shorter at 10 to 50 μM. Thus it appeared that both nicotine isomers may be exhibiting noncompetitive ion-channel-blocking properties simultaneously with their agonist properties.

Kinetics of activation of AChR by a combination of ACh and nicotine. ACh (0.4 μM) was combined with various concentrations of (-) and (+) nicotine in the patch pipet. Because ACh commonly causes isolated openings, it could have been possible to observe channel blockade by nicotine concentrations where channels were activated by ACh. Increasing concentrations of nicotine produced a dose-dependent decrease in the open time of single channel currents. Burst times dropped from the control level to that observed with nicotine isomers alone.

The nicotine isomers appear to have multiple effects on nicotinic synaptic function.

i.   Both (-) and (+) nicotine clearly act as agonists, although (-) is 8-10 times more potent than (+) nicotine in eliciting contracture and depolarizing the endplate.

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ii. The nicotine isomers both inhibited indirect twitch, (-) again being the more potent isomer. Intermediate concentrations of nicotine decreased the decay time constant of endplate currents (Fig. 20) and the mean channel open time in a manner suggestive of open channel blockade.

iii. During repetitive stimulation, the response was neither increased (ACh overcoming a nicotine-competitive blockade) nor decreased (allosteric or high-agonist desensitization) by up to 20 μM of either isomer (Fig. 18). In contrast, a noncompetitive desensitizing agent such as perhydro-histrionicotoxin, which allosterically modifies agonist affinity and channel activation, would have selectively decreased the amplitude of EPC responses in a frequency-dependent manner. Typical rundown in EPC amplitude in a train would be seen with high (desensitizing) concentrations of agonists. On the other hand, in the presence of a competitive antagonist such as curare, repetitive stimulation would have increased the final response by effective ACh competition. The blockade without frequency-dependent rundown may result from a slow inactivation caused by long-lasting application of low concentrations of agonist occurring simultaneously with noncompetitive antagonism at ion channel site(s).

iv. Above 100 μM, however, (-) nicotine alone caused a desensitized pattern of channel activation resembling that seen with high concentrations of agonists (Fig. 19). Intracluster openings were markedly shortened, leading to an apparent decrease in the amplitude of the current.

Considering the three mechanisms, it appears that for (-) and (+) nicotine the potency order is: agonist > noncompetitive channel blockade > desensitization, in contrast to the order for ACh which is agonist > desensitization > noncompetitive blockade.

D. SUMMARY AND CONCLUSIONS:

This project involving electrophysiological, toxicological and morphological studies provided data regarding protection afforded by carbamates and oximes against OPs both in vivo and in vitro. In addition, the electrophysiological studies offered a detailed description of the mode of action of various OPs, pyridinium compounds, carbamates and related agents at the peripheral nicotinic acetylcholine receptor ion channel. An attempt was also made to characterize the central nicotinic receptors using hippocampal, brainstem and retinal tissues. As part of the latter issue, the agonistic properties of (+)anatoxin-a at the central receptors and the isomers of nicotine at peripheral receptors were also investigated. A comparative account of the various properties pertaining to AChR and AChE for the OPs, carbamates and pyridinium compounds is listed in Table 8 and the relevance of each of these properties to its toxic/therapeutic effects is discussed below.

i) Agonistic property: Most of the OPs studied exhibited a weak agonistic property. The role of this property in the lethal actions of OPs is unclear; it is postulated that this

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property may enhance the desensitization process at the AChR. Among the protective agents, only the carbamates show some agonistic property. Therefore it is unlikely that this action contributes to their therapeutic efficacy.

ii) <u>Type of unblock from channels:</u> All the pyridinium compounds and the carbamates exhibited blockade of the AChR channel at the open state whereas of the OPs, only VX had this property. This property may play a role in the protection shown by carbamates and oximes. It is speculated that the cycling of the AChR through the blocked and unblocked states may help prevent the receptor from going into a long-lived desensitized states. Therefore drugs exhibiting fast and medium unblocking rates can keep the receptor functioning even during blockade. On the other hand, drugs with slow unblocking rates though may protect against desensitization, may themselves reduce the receptor activity below the level required for normal excitation.

iii) <u>Effect on channel opening probability</u>: All the four OPs decreased the channel opening probability whereas the pyridinium compounds either increased or left this parameter unchanged. (-) Physostigmine had minimal effect whereas (+) physostimine decreased the frequency of openings. A decrease in the open probability could arise from a competitive type blockade, a closed channel blockade, open channel blockade with very slow unblocking rates or due to desensitization process. In the case of OPs, the desensitization appears to play a significant role as it has been demonstrated for sarin. In the case of (+) physostigmine, the very slow unblocking may suffice to explain the results. In the case of oximes 2-PAM and HI-6, an increase in the open probability was observed which can either be due to an increase in the channel opening rate or to prevention of occurrence of agonist-induced desensitization. Whichever may be the reason, this effect of oximes would be beneficial for restoring the function of paralyzed muscles.

iv) <u>AChE-inhibition/ AChE-like action:</u> All the four OPs are potent inhibitors of AChE; by virtue of that they enhance the synaptic levels of ACh and thereby cause desensitization, in addition to that caused by other mechanisms. There appears to be no correlation between the inhibition of AChE and protection because oximes did not exhibit this property at therapeutically effective concentrations and moreover, (+)physostimine which was a weak inhibitor also exhibited protection against OPs except soman. However, the AChE-like action shown by the oximes may have some relevance to their therapeutic action. Under conditions of OP poisoning when the ACh-hydrolyzing ability is lost due to inhibition of AChE, the oximes can directly interact with the transmitter and terminate its action.

In conclusion, our studies provide insights into molecular mechanisms underlying the antidotal properties of the carbamates, oximes and non-oxime related compounds against lethal effects of irreversible AChE inhibitors. The data disclosed that carbamylation or reactivation of phosphorylated AChE is not the primary mechanism responsible for the antidotal properties of these agents against OPs. (+) Physostigmine's results from ultra-structural and <u>in vivo</u> toxicological studies provided the ultimate evidence for this theory. Moreover, the electrophysiological data showed that carbamates' protecting potency was strongly related to specific interactions with the molecular targets at the postsynaptic nicotinic AChR. Regarding the actions of oximes, studies on SAD-128 showed definite

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correlation between the antidotal efficacy of these compounds and their actions at the AChR macromolecule. Furthermore, our studies suggested that the direct interactions of OPs with nicotinic AChR targets (46,47) should be taken into account in the investigations of the carbamate-OP and oxime-OP antagonisms.

TABLE 8

AChR and AChE Modulatory Effects of Various Agents

Agent	Agonistic Action Yes/No	Type of unblock (Fast, medium, slow, very slow)	Channel open probability ($\uparrow,\downarrow,--$)	AChE-inhibition(A)/ AChE-like action(B)
VX	Yes	Very slow	$\downarrow$	A
Soman	Yes	No block	$\downarrow$	A
Sarin	Yes	No block	$\downarrow$	A
Tabun	?	No block	$\downarrow$	A
2-PAM	No	Fast	$\uparrow$	B
HI-6	No	Medium	$\uparrow$	B
SAD-128	No	Slow	--	None
(-)Phy	Yes	Fast	--	A
(+)Phy	Yes	very slow	$\downarrow$	A (weak)

? = under investigation; $\uparrow$ = increase; $\downarrow$ = decrease; -- = no change

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